II. REMARKS

A. Introduction

Applicants submit this Response in a bona fide attempt to (i) advance the prosecution of this case, (ii) answer each and every ground of objection and rejection as set forth by the Examiner, (iii) place the claims in a condition for allowance, and (iv) place the case in better condition for consideration on appeal.

Claims 1-29 are presently pending in the application. As indicated above, Claims 1-5 and 7 has been amended and Claims 6 and 18-29 have been cancelled. Claims 18-17 has previously been withdrawn.

Applicants respectfully submit that the noted amendments merely make explicit that which was (and is) disclosed or implicit in the original disclosure. The amendments thus add nothing that would not be reasonably apparent to a person of ordinary skill in the art to which the invention pertains.

B. Response to Rejections

1. Claim Amendments and Support Therefore

As indicated above, Claim 1, as amended, is based on pending Claim 24 (now cancelled), i.e. the preamble of Claim 24 has been incorporated into Claim 1. The limitation directed to "administration of a differential factor selected from the group consisting of IGF-II, a precursor of IGF-II, an isomer of IGF-II and an analog of IGF-II" has also been deleted and the limitation directed to "administration of an effective amount of IGF-II to a pregnant female mammal in the first half of pregnancy" has been substituted therefore.

Support for Claim 1, as amended, is set forth in the specification, as originally filed, e.g., Example 4 discloses administration of IGF-II to a pregnant female mouse in the first half of pregnancy. Support can also be found in original Claim 5.

Claim 2, as amended, reflects that the "effective amount of IGF-II" comprises an amount sufficient to promote binding of the IGF-II to a cation independent mannose 6 phosphate receptor expressed on a cytotrophoblast cell." Support for Claim 2, as amended, is also set forth in the specification, see, e.g., pp.10-13.

Claims 3-5, as amended, are directed to administration of IGF-II by subcutaneous delivery and/or vaginal pessary. Support for Claims 3-5, as amended, can be found in the specification, as originally filed, and in pending Claim 18. For example, Example 4 provides support for the administration of IGF-II via subcutaneous delivery. The use of vaginal pessaries is disclosed on page 13, line 33 of the specification.

Claim 7, as amended, is directed to the pregnant female mammal being selected from the group consisting of a human, a horse, a cow, a pig, a goat and a sheep. Support for Claim 7 can also be found in the specification, as originally filed, and in pending Claim 7. For example, page 7, lines 12 and 13 of the specification provides suitable mammalian species.

2. 35 U.S.C. §112

The Examiner has rejected Claim 24, which is now embodied in amended Claim 1, under 35 U.S.C. §112, second paragraph, "as being indefinite for failing to particularly point out and distinctly claim the subject matter which the application regards as the invention." The Examiner contends that Claim 24 (now amended Claim 1) does not recite "what the effective amount of the differential factor is supposed to achieve."

As indicated above, Claim 1, as amended, now reflects that administration of an effective amount of the differential factor, i.e. IGF-II, improves a physiological characteristic selected placental growth, placental development and placental differentiation.

3. 35 U.S.C. §102

The Examiner has also rejected Claim 24 (now amended Claim 1) under 35 USC §102(b) as being anticipated by U.S. Pat. No. 5,420,111. The Examiner contends that U.S. Pat. No. 5,420,111 teaches a method of administration of IGF-II to a pregnant female at "any time from conception onward".

It is well established that a rejection for anticipation under § 102 requires that each and every limitation of the claimed invention be disclosed in a single prior art reference. *See In re Paulsen*, 30 F.3d 1475, 1478-79, 31 U.S.P.Q. 2d 1671, 1673 (Fed. Cir. 1994); *Scripps Clinic & Research Foundation v. Genentech, Inc.*, 927 F.2d 1565, 18 U.S.P.Q. 2d 1001 (Fed. Cir.1991). *See also American Permahedge, Inc. v. Barcana, Inc.*, 857 F. Supp. 308, 32 U.S.P.Q. 2d 1801, 1807-08 (S.D. NY 1994) ("Prior art anticipates an invention ... if a single prior art reference contains each and every element of the patent at issue, operating in the same fashion to perform

the identical function as the patent product. ... Thus, any degree of physical difference between the patented product and the prior art, *no matter how slight*, defeats the claim of anticipation."); *Transco Ex parte Levy*, 17 U.S.P.Q. 2d 1461, 1462 (Bd. Pat. App. & Int'l 1990) ("[I]t is incumbent upon the examiner to identify wherein each and every facet of the claimed invention is disclosed in the applied reference".)

Applicants respectfully submit that Claim 1, as amended, and Claims 2-5 and 7, dependent thereon, are not anticipated by U.S. Pat. No. 5,420,111.

U.S. Pat. No. 5,420,111 discloses administration of IGF-I to a pregnant mammal to promote fetal growth. The '111 patent does not disclose that the administration of IGF-II (or IGF-I) improves placental growth, development or differentiation.

In support of the contention that U.S. Pat. No. 5,420,111 discloses administration of IGF-II to a pregnant female mammal, the Examiner relies on the statement in the '111 patent that "although the studies to be discussed herein concentrate on the use of IGF-I, the claims extend to IGF-II and analogues of IGF-I and IGF-II as these are known to exert a similar biological effect to IGF-I (Schoenle et al., Acta Endoc. 108: 167-174, 1985)."

However, it is submitted that one skilled in the art would recognize that the biological effects of IGF-II are ***quite different*** to that of IGF-I (see, e.g., Fowden A. L., "The Insulin-like Growth Factors and Feto-Placental Growth", *Placenta, vol.* 24, pp. 803-812 (2003) and Sferruzi-Perri, et al., "Maternal Insulin-Like Growth Factors-I and –II Act via Different Pathways to Promote Fetal Growth", *Endocrinology*, vol. 147(7), pp. 3344-3355 (2006), copies attached). Thus, one skilled in the art would recognize that while the '111 patent discloses that treatment of IGF-I to a pregnant female mammal may extend to analogues of IGF-I, one skilled in the art would also recognize that the disclosure does not extend to IGF-II.

Applications further submit that U.S. Pat. No. 5,420,111 does not disclose when or how to administer IGF-II to a pregnant female to improve placental growth, placental function, placental development or placental differentiation. Further, the '111 patent does not teach or suggest that the administration of IGF-II to improve placental weight, development or differentiation.

The U.S. Pat. No. 5,420,111 merely discloses that the compositions may be administered "at any time from conception onward" (column 2, last paragraph). Indeed, the '111 patent

discloses that IGF-I is "[d]esirably administered close to the time of birth of the fetus" (column, last paragraph), which teaches away from amended Claim 1.

In addition, given the lack of teaching in U.S. Pat. No. 5,420,111 as to how and when to administer IGF-II, one skilled in the art would recognize that an improvement in placental growth, development or differentiation would not necessarily flow based on the teaching provided in the '111 patent.

U.S. Pat. No. 5,420,111 also teaches away from the current invention by stating that IGF-I has no effect on placental weight (see Example 1). The '111 patent further discloses in Example 3 that since IGF-I does not cross that rat placenta, the effect of IGF-I is clearly in the maternal compartment (see column 8, 3rd paragraph).

Applicants therefore respectively submit that Claim 1, as amended, is not anticipated by U.S. Pat. No. 5,420,111.

III. CONCLUSION

Applicants, having answered each and every ground of rejection as set forth by the Examiner, and having added no new matter, believe that this response clearly overcomes the references of record and renders the claims clear and definite, and now submit Claims 1-5 and 7 in the above-referenced patent application are in condition for allowance and the same is respectfully solicited.

If the Examiner has any further questions or comments, Applicants invite the Examiner to contact their Attorneys of record at the telephone number below to expedite prosecution of the application.

Respectfully submitted,
FRANCIS LAW GROUP

By: _____
Ralph C. Francis
Reg. No. 38,884

Dated: January 8, 2007
1942 Embarcadero
Oakland, CA 94606
Tel: 510.533.1100

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CURRENT TOPIC

The Insulin-like Growth Factors and feto-placental Growth

Abigail L. Fowden*

Department of Physiology, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK

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The insulin-like growth factors, IGF-I and IGF-II, have an important role in fetoplacental growth throughout gestation. They have metabolic, mitogenic and differentiative actions in a wide range of fetal tissues including the placenta. Both *Igf1* and *Igf2* genes are expressed in fetal tissues. Expression of the *Igf2* gene is more abundant than Igf1 gene expression during mid to late gestation. Both IGF's are also present in the fetal circulations with 3–10 fold higher levels of IGF-II than IGF-I during late gestation. Expression of the *Igf* genes is developmentally regulated in a tissue specific manner and can be affected by nutritional and endocrine conditions *in utero*. Deletion of either *Igf* gene of the *Igf1r* gene retards fetal growth while over-expression of IGF-II leads to fetal overgrowth. In mice, placental growth is affected only by manipulation of the *Igf2* gene. The IGF's also effect the growth of individual fetal tissues and influence the uptake and utilization of nutrients by the fetal and placental tissues. Circulating concentrations and tissue expression of the IGF's are reduced by undernutrition and deficiency of nutritionally sensitive hormones, such as insulin, thyroxine and glucocorticoids. In general, the *Igf1* gene is more responsive to these stimuli than the *Igf2* gene. In addition, the effects of the IGFs on feto-placental growth can be amplified or attenuated by the IGF binding proteins, which are themselves regulated by nutritional and endocrine signals. The *Igf2* gene appears to provide the constitutive drive for intrauterine growth via its placental effects and direct paracrine actions on fetal tissue while the *Igf1* gene regulates fetal growth in relation to the nutrient supply.

Placenta (2003), 24, 803–812

INTRODUCTION

The insulin-like growth factors, IGF-I and IGF-II, have a key role in regulating feto-placental growth throughout gestation. They have metabolic, mitogenic and differentiative actions in a wide range of fetal tissues including the placenta (Jones and Clemmons, 1995). They act as progression factors in the cell cycle and increase DNA synthesis and cell differentiation in cultured embryos and several different fetal cell lines in vitro (Han and Fowden, 1994; Gardner et al., 1999). Their concentrations in the fetus in vivo are positively correlated to birth weight in a number of species including humans, primates, sheep, pigs, rabbits and rodents (Daughaday et al., 1982; Gluckman et al., 1983; Lee, Chung and Simmen, 1993; Tarantal and Gargosky, 1995; Kind et al., 1995; Thakur et al., 2000; Ong et al., 2000). This review examines the relationship between the IGFs and feto-placental growth and places particular emphasis on the expression, action and regulation of the IGFs in fetal and placental tissues. It considers the insulin-like growth factor binding proteins (IGFBPs) in much less detail as their regulation and role in modulating the actions of the IGFs have been reviewed recently (Allan, Flint and Patel, 2001; Schneider et al., 2002; Mohan and Baylink, 2002).

EXPRESSION OF THE IGFS BEFORE BIRTH

In many species, both the *Igf1* and *Igf2* genes are expressed in fetal tissues from the earliest stage of pre-implantation development to the final phase of tissue maturation just before birth (Watson et al., 1994; Hill, Petrik and Arany, 1998; Fowden, Li and Forhead, 1998). During mid to late gestation, *Igf2* gene expression is widespread in fetal tissues and is more abundant than *Igf1* gene expression in rodents, ungulates and humans (Hill, 1990; Delhanty and Han, 1993). Both IGFs are also detected in the fetal circulation from early in gestation but plasma concentrations of IGF-II are 3–10 fold higher than those of IGF-I during late gestation in all species studies so far (Table 1). Tissue and plasma IGF-II are also higher in the fetus than in newborn or adult animals in most species (Gluckman and Butler, 1983; Mesiano et al., 1987). In rodents, IGF-II expression disappears from most tissues except the brain by weaning, with the consequence that IGF-II is virtually undetectable in adult plasma (Lee, Lintar and Efstratiadis, 1990; Singh, Rall and Styne, 1991). In ungulates, *Igf2* gene

* To whom correspondence should be addressed. Tel.: +44-1223-333855; fax: +44-1223-333840; E-mail: alf1000@cam.ac.uk

0143-4004/03/$–see front matter

Table 1. Fetal plasma concentrations of IGF-I and IGF-II during late gestation in different species

	Plasma concentrations (ng/ml)		
	IGF-I	IGF-II	Reference
Human	50–100	150–400	Gluckman et al., 1983
Monkey	70–80	300–400	Tarantal & Gargosky, 1995
Sheep	50–100	400–1000	Owens et al., 1994
Cattle	50–80	280–360	Holland et al., 1997
Pig	20–30	200–300	Lee, Chung and Simmen, 1993
Guinea pig	50–100	500–100	Jones et al., 1987
Rat	50–100	400–700	Daughaday et al., 1982

expression is retained in certain peripheral tissues, such as skeletal muscle after birth and, hence, IGF-II is present in the adult circulation, albeit at lower concentrations than in the fetus (Mesiano et al., 1987; Lee, Chung and Simmen, 1993; Holland et al., 1997). In contrast, tissue expression and plasma level of IGF-I are low in utero compared to postnatal values (Gluckman and Butler, 1983; Mesiano et al., 1987; Singh, Rall and Styne, 1991). Plasma IGF-I levels increase rapidly after birth, primarily as a result of the onset of growth hormone (GH) stimulated IGF-1 production by the liver (Gluckman, 1995; Li et al., 1999). There is, therefore, a shift in IGF predominance from IGF-II before birth to IGF-I after birth, which has led to the concept that IGF-II is the IGF primarily responsible for fetal growth (see Gluckman, 1995; Jones and Clemmons, 1995; Allan, Flint and Patel, 2001).

Abundance of the IGF mRNAs varies widely between different fetal tissues and with gestational age. In the sheep fetus, for instance, *Igf2* gene expression is particularly high in the lung and kidney while IGF-I mRNA abundance is highest in liver and skeletal muscle (Delhanty and Han 1993; Kind et al., 1995). Similar differential patterns of IGF expression have also been observed in fetal tissues from rodents and human and non-human primates (Hill, 1990; Lee, Lintar and Efstratiadis, 1990; Lee et al., 2001). The developmental changes in IGF expression are also tissue and IGF specific. In fetal sheep, *Igf1* gene expression is up- and down regulated during late gestation in liver and skeletal muscle, respectively (Fig. 1), while *Igf2* gene expression is suppressed in these tissues and the adrenal, although not in the lung and kidney towards term (Li et al., 1993, 1996; Li et al., 1994; Forhead et al., 2002). The switch from widespread local production of IGF before birth to a more selective pattern of expression after birth, therefore, begins during late gestation before delivery actually occurs. With the transition from parenteral to enteral nutrition at birth, the perinatal switch from local production of predominantly IGF-II to GH dependent production of IGF-I contributes to the resetting of the growth regulatory mechanisms that ensure continued postnatal growth in the new nutritional environment.

In the placenta, expression of the IGFs is species specific. The rodent placenta expresses only the *Igf2* gene while the placenta of guinea pigs, ungulates, human and non-human primates express both *Igf* genes (Lee, Lintar and Efstratiadis, 1990; Lennard, Stewart and Allen, 1995; Han and Carter, 2000). In the latter species, the two IGFs are often localized to specific placental tissues (Lee, Lintar and Efstratiadis, 1990; Han and Carter, 2000). In sheep, IGF-II mRNA is found primarily in fetal mesoderm within the placentomes while IGF-I mRNA is confined to the uterine glands in the inter-cotyledonary regions (Wathes et al., 1998). In general, IGF-II is expressed in fetal tissue at the fetal-maternal interface of the placenta and in the invading trophoblast in species with invasive placentation (Han and Carter, 2000). Much less is known about the developmental changes in IGF expression in placental than fetal tissues but increased expression of IGF-II has been observed in syncytiotrophoblast and whole villous tissue of primates with increasing gestational age (Zollers et al., 2001). In ruminants, the placenta is both a source of fetal plasma IGF-II and a site for IGF-I clearance from the fetal circulation (Bassett et al., 1998; Holland et al., 1997).

Each of the *Igf* genes has several promotors which leads to multiple mRNA transcripts with different 5' and 3' untranslated regions (Dickson, Saunders and Gilmour, 1991; Gilmour, 1994). These splice variants show developmental and tissue-specific patterns of expression in the fetus (Adamo et al., 1989; Li et al., 1996; Lin and Oberbauer, 1998; Constancia et al., 2000). In sheep, the IGF-I mRNA transcripts are classified as Class 1 or Class 2 depending on whether they are derived from 5' leader exons 1 or 2 (Gilmour, 1994). In adult liver, Class 2 transcripts predominate whereas, in fetal liver, Class 1 is the primary transcript for most of late gestation with little, if any, Class 2 expression until just before term (Figure 1). Similarly, the *Igf2* gene is expressed from at least two promotors in utero in a manner which is tissue specific and dependent on gestational age (Li et al., 1998; Constancia et al., 2000). The *Igf2* gene is also imprinted and expressed only from the paternal allele in the placenta and several fetal tissues excluding the brain (Ferguson-Smith et al., 1991; Miozzo and Simoni, 2002). However, after birth, *Igf2* expression becomes biallelic in tissues, such as the liver, in a number of species including sheep, cattle and humans, although not in mice (DeChiara, Robertson and Efstratiadis, 1990; Kalscheuer et al., 1993; Davies, 1994; McLaren and Montgomery, 1999). Imprinting of *Igf2* is controlled by the *H19* gene, which is itself imprinted and developmentally regulated (Senior et al., 1996; Naimeh et al., 2001). Consequently, there are ontogenic shifts in *Igf2* imprinting and *IGF* gene promotor usage which may influence IGF bioavailability in placental and fetal tissues at critical stages of development.

THE ACTIONS OF THE IGFS ON TISSUE GROWTH AND DEVELOPMENT IN UTERO

In recent years, manipulation of gene expression in mice has been used widely to establish the role of the IGFs in

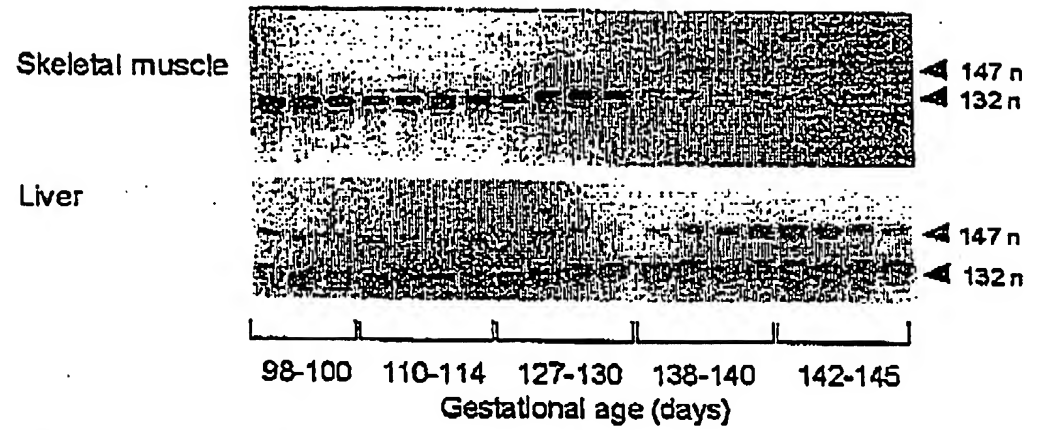

Figure 1. The ontogeny of IGF-1 gene expression in fetal ovine tissues during late gestation. Autoradiograms of RNAase protection assay using ovine IGF-I riboprobes with 50 μg total RNA prepared from liver and skeletal muscle of groups of control sheep fetuses aged 100–145 days of gestation (term 145 ± 2 days). Protected probes gave bands at 132 nucleotides (132n) for Class 1 transcripts and at 147 nucleotides (147n) for Class 2 transcripts of the *Igf1* gene. Data from Li et al., 1996, 2002.

Table 2. The effects of disruption of genes controlling IGF bioavailability on fetal and placental weights in mice during late gestation (>85%)

Gene target	Effect	Per cent of normal weight		Reference
		fetus (%b)	Placenta (%b)	
Igf1	No tissue or plasma IGF-I	60	100	Baker et al., 1993
Igf2	No tissue or plasma IGF-II	60	75	DeChiara, Robertson and Efstratiadis, 1990
Placental P0 *Igf2*	Decrease placental IGF-II. Normal fetal IGF-II	75	65	Constancia et al., 2002
IGF-type 1 receptor (*Igf1r*)	No action of IGF-I/IGF-II at IGF1r	45	100	Baker et al., 1993
GF-type 2 receptor (*Igf2r*)	No IGF-II clearance. Increased plasma IGF-II	140	140	Ludwig et al., 1996
H19	No suppression of maternal *Igf2* allele. Increased tissue IGF-II	130	140	Lau et al., 1994
Igf2r and *H19*	Increased tissue and plasma IGF-II	200	230	Eggenschwiler et al., 1997

feto-placental growth (Efstratiadis, 1998). Deletion of either the *Igf1* or *Igf2* gene retards fetal growth to a similar extent (Table 2). When both genes are deleted simultaneously, the effects on fetal growth are additive and the double mutants are only 30 per cent of the normal bodyweight at term (Efstratiadis, 1998). Deletion of the IGF-type 1 receptor gene (*Igf1r*) produces a more severe growth retardation than seen in either the *Igf1* or *Igf2* nulls (Table 2) which suggests that both IGFs act through the type 1 IGF receptor to stimulate tissue accretion (Efstratiadis, 1998). Conversely, fetal growth is enhanced by IGF-II over-expression caused either by deletion of the IGF-type 2 clearance receptor (*Igf2r* null) or by biallelic IGF-II expression in response to *Igf2* imprint relaxation induced by disruption of the *H19* gene (Table 2; Lau et al., 1994; Ludwig et al., 1996). fetal overgrowth is greatest in the double *Igf2r/H19* mutants, which have the highest IGF-II levels and the largest placentae (Table 2; Eggenschwiler et al., 1997). In the human, homozygous partial deletion of the IGF-I gene is also associated with failure of growth, both in utero and postnatally (Woods et al., 1996).

These IGF-induced changes in fetal bodyweight are accompanied by abnormalities in the development of individual fetal tissues (Woods et al., 1996; Efstratiadis, 1998). The *Igf1* and *Igf2* null mice were both viable although they showed delayed ossification and general dwarfisms at birth. The growth rate of the *Igf1*, but not *Igf2* null mice remained low after birth which is consistent with the loss of IGF-II expression in wild types after weaning (see Miozzo and Simoni, 2002). Deletion of the IGF type 1 receptor had more widespread effects on murine tissue growth and lead to delayed ossification, thin skin and hypoplasia of respiratory and other muscles, which proved fatal at birth (Efstratiadis, 1998). Over-expression of IGF-II caused generalized organomegaly with kinky tails, extra toes, oedema and cardiac abnormalities

and was usually lethal at birth (Lau et al., 1994; Louvi, Accili and Efstratiadis, 1997). Similarly, in sheep produced in vitro or by cloning, increased IGF-II exposure induced by reduced *Igf2r* gene expression is associated with multiple developmental abnormalities, muscle hypertrophy and generalized overgrowth of the fetus (Young et al., 2001).

In mice, placental growth is affected by manipulation of the *Igf2*, but not the *Igf1* or *Igf1r* genes (Table 2). The placenta is growth retarded by 30–40 per cent in mice that lack IGF-II either in all placental cell types (*Igf2* null, DeChiara, Robertson and Efstratiadis, 1990) or in the labyrinthine trophoblast cells specifically (P0 null, Constancia et al., 2002). In P0 mutants, the placenta is small but morphologically normal whereas, in *Igf2* nulls, placental growth retardation is accompanied by structural abnormalities, particularly in the glycogen cells (Rossant and Cross, 2001; Constancia et al., 2002). Conversely, placentomegaly occurs when IGF-II is over-expressed by changes in IGF-II clearance or *Igf2* imprinting (Table 2). The growth stimulatory effects of IGF-II on the placenta may be paracrine and/or endocrine but do not appear to be mediated via the IGF type 1 receptor (Table 2). Placental growth is also normal in double mutants lacking both IGF type 1 and insulin receptors which suggests the IGF-II may act through an unknown placental specific receptor (Louvi, Accili and Efstratiadis, 1997). The existence of another type of IGF receptor in the placenta may also explain the unusual characteristics of IGF-I binding observed in the ovine trophoblast between 45–75 days of gestation when no *Igf1r* gene expression can be detected in the placentomes (Lacroix, Servety and Kann, 1995; LeRoith et al., 1995; Wathes et al., 1998). However, whether this placental specific IGF receptor is responsible for placentomegaly in mice during IGF-II over-exposure remains unknown.

In *Igf2* nulls, placental and fetal growth retardation occurs in parallel and begins around mid gestation (Baker et al., 1993). In P0 mutants lacking IGF-II only in the labyrinthine placenta, growth retardation of the placenta begins at a similar stage but growth of the fetus is not slowed until much later in gestation (Constancia et al., 2002). At term, the weight of the fetus produced per gram of placenta was greater in P0 mutants than in wild types although both the P0 placenta and fetus were smaller than normal at this stage. These observations suggest that IGF-II may affect the functional capacity of the placenta to transfer nutrients as well as placental size. Both IGFs have been shown to alter glucose and amino acid transfer across cultured human trophoblast derived from chorionic villi (Kniss et al., 1994). Similarly, administration of IGF-I to either the fetus or mother has been shown to alter the transfer and partitioning of glucose and amino acids between ovine fetal and uteroplacental tissues (Harding et al., 1994; Liu et al., 1994). Changes in expression of the amino acid transporter proteins have been observed in specific regions of the *Igf2* null placenta (Matthews et al., 1999). Measurement of passive and secondarily active transport across the P0 mutant placenta has shown that passive diffusion is reduced while System A amino acid transport is increased per unit surface area of placenta throughout late gestation (Constancia et al., 2002). Up-regulation of System A amino acid transport, therefore, appears to compensate for the smaller size of the P0 placenta for much of gestation and only fails to meet the growth requirements of the fetus late in gestation (Reik et al., 2003). Whether this up-regulation of amino acid transport is the consequence of a paracrine IGF-II deficiency in the labyrinthine placenta or of an endocrine action of the normal circulating levels of the IGF-II in the P0 fetus has yet to be determined.

While gene manipulation experiments have shown that IGF-I affects fetal growth directly, they suggest that the growth-promoting actions of IGF-II on the fetus may be indirect and mediated via changes in the growth and nutrient transport capacity of the placenta (Table 2). However, more detailed comparison of the growth rates of various IGF mutants has shown that fetal growth is determined by the actions of IGF-I on the IGF type 1 receptor and of IGF-II on both the IGF type 1 and insulin receptors (Eggenschwiler et al., 1997; Louvi, Accili and Efstratiadis, 1997; Efstratiadis, 1998). The growth-promoting action of IGF-II was predominantly through the IGF type 1 receptor, although insulin receptor mediated action increased during late gestation to account for about 40 per cent of the total IGF-II activity at term (Louvi, Accili and Efstratiadis, 1997). The interactions of IGF-I and IGF-II with the IGF type 1 receptor were equally as important in determining fetal growth during late gestation (Baker et al., 1993).

Administration of IGF-I directly to sheep and monkey fetuses for 10 days has no effect on placental or fetal body weight (Lok et al., 1996; Tarantal, Hunter and Gargosky, 1997). However, in both species, IGF-I increased the weight of specific fetal organs such as the spleen, thymus and kidney. It also increased the weight of the liver, lungs, heart, pituitary and adrenal glands in the sheep fetus (Lok et al., 1996). In addition, IGF-I administration promoted skeletal maturation in the sheep fetus during late gestation (Lok et al., 1996). More long-term administration of IGF-I via the gut (30 days) has been shown to increase total bodyweight in growth retarded sheep fetuses (Kimble et al., 1999). These changes in growth of the internal organs and skeleton are probably the result of the anabolic actions of IGF-I on fetal metabolism. Short-term infusion of IGF-I (4 h) into the sheep fetus has been shown to increase placental amino acid transfer and to decrease proteolysis and amino acid oxidation in fetal tissues (Harding et al., 1994; Boyle et al., 1998; Jensen et al., 2000). This would increase the availability of amino acids for protein synthesis and the accretion rate of protein in the fetal carcass. However, IGF-I administration reduces the fetal plasma concentration of insulin (Leichty et al., 1996), a major promoter of fetal growth (Fowden, 1995). It also suppresses *Igf1* and *Igf2* gene expression in fetal ovine liver which may reduce the paracrine stimulus for tissue growth (Kind et al., 1996). Changes in insulin secretion and local IGF production may therefore explain the selective effects of IGF-I administration on tissue growth in sheep and monkey fetuses.

Table 3. The effects of manipulating the fetal nutrient supply on fetal IGF concentrations

Treatment	Species	Per cent change in plasma IGF		Reference
		IGF-I (%)	IGF-II (%)	
Maternal nutrition				
Protein deprivation	Rat	↓50–60	No change	Muaku et al., 1995
Fasting	Rat	↓60–70	↓10	Straus et al., 1991
	Sheep	↓50	↓15–20	Oliver et al., 1996; Lee et al., 1997
Restrict uterine blood flow	Rat	↓50	No change to ↑10	Price et al., 1992
	Guinea pig	↓70	No change	Jones et al., 1987
	Sheep	↓50	↓20	McLellan et al., 1992
Restrict placental function				
Caruncelectomy	Sheep	↓70–75	No change to ↓20	Owens et al., 1994
Cord occlusion—partial	Sheep	No change	No change	Green et al., 2000
—complete	Sheep	↓80	No change	Bennet et al., 2001
Maternal hypoxia	Rat	↓10	↑40	Tapanainen et al., 1994
	Sheep	↓40–50	No change	Iwamoto et al., 1992

As well as stimulating cell proliferation, IGF-I and IGF-II have been shown to prevent apoptosis in cultured cell lines (Han and Fowden, 1994; Allan, Flint and Patel, 2001). In rodents, the β cells of the endocrine pancreas undergo programmed apoptosis followed by a wave of islet neogenesis around the time of weaning (see Hill, Petrik and Arany, 1998). This sequence of β cell destruction and renewal coincides with a decrease in pancreatic *Igf2* gene expression and with a switch from fetal β cells capable of replication to non-proliferating β cells with insulin secretory responses characteristic of the adult (see Fowden and Hill, 2001). When IGF-II levels are maintained during weaning by transgenic over-expression of IGF-II, the wave of apoptosis does not occur and β cell mass increases five fold (Hill, Petrik and Arany, 1998). These observations suggest that IGF-II may have a key role in cell differentiation, particularly during the perinatal period when many tissues are adapting to new environmental conditions. Certainly, in the sheep fetus, the decline in *Igf2* gene expression in the liver, muscle and adrenal towards term coincides with the main phase of prepartum structural and functional maturation in these tissues (Li et al., 1993, 1996, 2002; Lü et al., 1994).

REGULATION OF IGF EXPRESSION

Nutritional regulation

fetal IGF concentrations are affected by a wide range of experimental manipulations which alter the placental supply of nutrients to the fetus (Table 3). Reduced availability of both substrates and oxygen or of either substrate or oxygen alone lower fetal IGF concentrations (Table 3). Nutrient restriction has a more pronounced effect on circulating levels of IGF-I than IGF-II, irrespective of the cause or nature of the nutrient deficit (Table 3). Similarly, there is a greater reduction in tissue abundance of IGF-I than IGF-II mRNA during nutrient restriction in fetal rats and sheep (Strauss et al., 1991; Kind et al., 1996; Brameld et al., 2000). In fetal sheep, IGF-I, but not IGF-II concentrations are directly correlated with the fetal arterial blood pO_2 and glucose levels during late gestation (Carr et al., 1995). Indeed, IGF-I levels can be raised in the fetus of fasted ewes by direct fetal infusion of either glucose or insulin (Oliver et al., 1996). Since insulin increases glucose uptake by fetal tissues (Fowden, 1995), these observations suggest that IGF-I is regulated by the cellular availability of glucose (Fowden, Li and Forhead, 1998). In contrast, fetal levels of IGF-II are reduced only during the severest types of growth retardation or when nutrient deprivation is particularly extreme or prolonged (Owens et al., 1994; Holmes et al., 1997). The *Igf1* gene, therefore, appears to be more responsive to changes in nutritional state than the *Igf2* gene in the fetus during late gestation. These observations are consistent with the findings that birth weight is more closely correlated with plasma IGF-I than IGF-II in several species (Carr et al., 1995; Ong et al., 2000).

Endocrine regulation

fetal IGF concentrations are also affected by the endocrine environment in utero, particularly by nutritionally sensitive hormones known to regulate fetal development, such as insulin, thyroxine and glucocorticoids (Fowden, 1995). Like nutrient restriction, deficiency of these hormones in utero affects expression of IGF-I more readily than IGF-II. Compared to the adult, GH has relatively little effect on the IGF axis in the fetus, probably due to the paucity of GH receptors in fetal tissues for most of gestation (Gluckman, 1995; Fowden, Li and Forhead, 1998). Insulin deficiency, on the other hand, reduces plasma IGF-I, but not IGF-II levels in the sheep fetus (Gluckman et al., 1987). Conversely, insulin infusion raises plasma IGF-I, but has no effect on IGF-II levels (Oliver et al.,

1996). fetal insulin and IGF-I levels are, therefore, positively correlated over the normal range of concentrations observed in utero and act synergistically to enhance accumulation of glucose and amino acid carbon, respectively, in the fetal tissues (Owens, 1991; Fowden, 1995; Han and Fowden, 1994).

In fetal sheep and pigs, circulating IGF-I, but not IGF-II concentrations are also reduced by thyroid hormone deficiency and are restored to normal values by thyroxine treatment (Mesiano et al., 1989; Latimer et al., 1993). The low levels of IGF-I induced by hypothyroidism were accompanied by fetal growth retardation (Fowden, 1995) and by tissue-specific changes in *Igf1*, but not *Igf2* gene expression (Latimer et al., 1993; Forhead et al., 1998, 2000). In fetal pigs, thyroid hormone deficiency reduced the IGF-I content of a wide range of fetal tissues, including the liver and skeletal muscle (Latimer et al., 1993). In contrast, thyroidectomy of the sheep fetus increased IGF-I mRNA levels in the liver, but reduced its abundance in skeletal muscle during late gestation (Forhead et al., 2000, 2002). Hypothyroidism also altered the normal ontogenic pattern of *Igf1* gene expression in both these tissues towards term (Forhead et al., 2000, 2002). Hence, thyroid hormone mediated changes in *Igf1* gene expression probably have an important role in regulating fetal growth, particularly in tissues, such as skeletal muscle, which normally accounts for 25–33 per cent of fetal bodyweight at term (Owens, 1991). However, the effects of thyroid hormones on placental development and *Igf* gene expression remain largely unknown.

In contrast to insulin and the thyroid hormones, glucocorticoids affect expression of both *Igf* genes, although their effects are tissue and IGF specific (Fowden, Li and Forhead, 1998). In fetal sheep, cortisol up- and down-regulates *Igf1* gene expression in liver and skeletal muscle, respectively, whereas, it down-regulates *Igf2* gene expression in these tissues (Figure 2). These changes in tissue expression occur both in response to exogenous cortisol infusion before term and when fetal cortisol levels rise endogenously during the immediate prepartum period (Figure 2). The cortisol induced changes in tissue *Igf* gene expression are also accompanied by decreases in the fetal growth rate and, close to term, by a fall in plasma IGF-II levels (Gluckman et al., 1983; Fowden et al., 1996). Cortisol, therefore, appears to initiate the switch from paracrine IGF production in utero to the hepatic production of endocrine IGF-I characteristic of the postnatal animal. However, the mechanisms by which cortisol acts remain unclear. Cortisol has been shown to suppress transcription of the ovine *Igf2* gene via specific promotors in fetal liver in vivo and in cell lines in vitro (Li et al., 1998). In contrast, the ovine *Igf1* gene contains no recognizable glucocorticoid response elements (Dickson, Saunders and Gilmour, 1991). Hence, cortisol may act on *Igf* gene expression either directly or indirectly through changes in GH receptor gene expression (Li et al., 1999) and/or via other transcription factors or cortisol-dependent hormones, such as triiodothyronine (Forhead et al., 1998, 2002). Whether the prepartum cortisol surge is also involved in the perinatal transition from monoallelic to biallelic *Igf2* gene expression remains unknown.

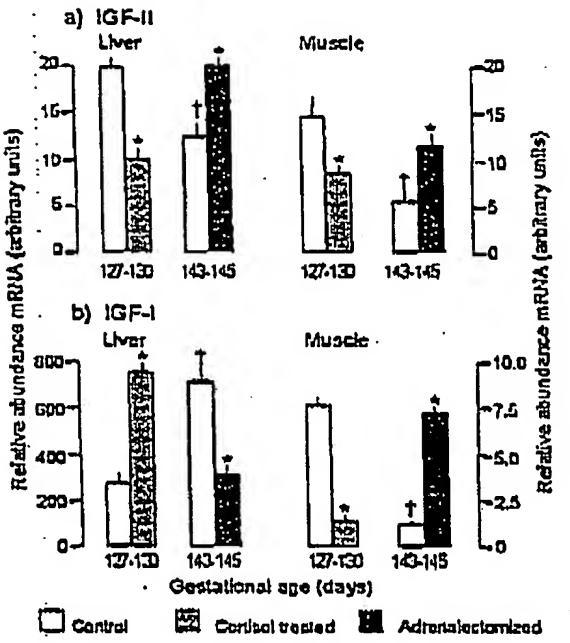

Figure 2. The control of IGF gene expression in fetal ovine tissues by cortisol during late gestation. Cortisol levels were manipulated before term by cortisol infusion and at term by fetal adrenalectomy. The figure shows mean (±SD) abundances of (a) IGF-II mRNA and (b) IGF-I mRNA in liver and skeletal muscle from sheep fetus delivered either before term (127–130 days) after 5 days of infusion of saline (open columns, controls n=5 low cortisol values) or cortisol (grey columns, 2-mg/kg/day, n=5, high cortisol values) or at (143–145 days) with (open columns, controls n=4, high cortisol values) or without adrenal glands (black columns, adrenalectomized n=4, low cortisol values). *Significantly different from value in the age-matched control group $P<0.05$, †significantly different from value in control fetuses at 127–130 days, $P<0.05$. Data from Li et al., 1998, 1996, 2002.

Increases in fetal plasma cortisol also occur before term during adverse intrauterine conditions, such as hypoxaemia and undernutrition (Challis et al., 2001). Although these increments tend to be smaller than those seen at term, they may explain, in part, the changes in tissue *Igf1* gene expression observed during nutrient restriction (Table 3). The ability of glucocorticoids to suppress *Igf2* gene expression in certain fetal tissues is also consistent with the observations that fetal IGF-II levels only fall close to term and during the severest types of growth retardation when fetal cortisol levels are high. Indeed, glucocorticoid-dependent changes in *Igf2* gene expression may be the major mechanism regulating IGF-II availability in the fetus during late gestation.

IGFBP regulation

The bioavailability of the IGFs is also affected by the tissue expression and circulating concentrations of the IGFBPs

(Jones and Clemmons, 1995) and of the soluble form of the IGF-II receptor, which binds up 40 per cent of the IGF-II in fetal ovine plasma (Gallaher et al., 1994). At least six different IGFBPs have been identified in fetal plasma and tissues, each of which has a unique pattern of expression (Jones and Clemmons, 1995; Allan, Flint and Patel, 2001). In rodents, ungulates, humans and non-human primates, the most prevalent IGFBPs in fetal plasma and tissue are the IGFBPs 1 to 4, although their relative abundance varies both within and between species (Donovan et al., 1989; Lee, Chung and Simmen, 1993; Carr et al., 1995; Kind et al., 1995; Tarantal and Gargosky, 1995; Osorio et al., 1996). fetal expression of these IGFBPs is also tissue specific and developmentally regulated in most species studied (Donovan et al., 1989; Delhanty and Han, 1993; Lee, Chung and Simmen, 1993; Carr et al., 1995; Tarantal and Gargosky, 1995).

In sheep and humans, fetal bodyweight at term is positively correlated to plasma IGFBP-3, but inversely related to plasma IGFBP-1 over the normal range of birthweights (Carr et al., 1995; Kind et al., 1994; Kajantie et al., 2001). When intrauterine growth is retarded in human infants, plasma concentrations of IGFBP-1 and -2 are elevated while IGFBP-3 levels are reduced compared to the values found in normally grown infants of the same gestational age (Lassarre et al., 1991; Chard, 1994; Ong et al., 2000). Similarly, hepatic expression and plasma levels of IGFBP-1 are increased in growth retarded rat pups during late gestation (Strauss et al., 1991; Price et al., 1992). Transgenic over-expression of IGFBP-1 and -3 in mice also retards growth, both pre- and post-natally (Silha and Murphy, 2002). Changes in IGFBP expression, therefore, have an important role in modulating the growth-promoting actions of the IGFs, although identifying the specific effects of each IGFBP is difficult because of their functional redundancy (Allan, Flint and Patel, 2001; Silha and Murphy, 2002).

During late gestation, IGFBP expression in the fetus is affected by both the nutritional and endocrine conditions in utero. Generally, these conditions have more pronounced effects on IGFBP-1, -2 and -4 than IGFBP-3. Tissue expression and plasma levels of IGFBP-1 are elevated in rat and sheep fetuses by fetal nutrient restriction induced by maternal dietary restriction, reduced uterine blood flow or by occlusion of the umbilical cord (Strauss et al., 1991; Price et al., 1992; Osborn et al., 1992; Hooper et al., 1994; Bennet et al., 2001). Conversely, increasing fetal glucose levels lowers hepatic expression and plasma IGFBP-1 in fetal sheep (Osborn et al., 1992). In contrast, levels of the soluble form of the IGF-II type 2 receptor are lowered by fetal undernutrition and raised by fetal hyperglycaemia (Gallaher et al., 1994). Specific fetal hypoxaemia has also been shown to increase IGFBP-1 levels in fetal ovine plasma (Iwamoto et al., 1992). Similarly, in human infants, IGFBP-1 levels are higher in hypoxic than normoxic neonates at birth (Chard, 1994). The increase in fetal IGFBP-1 expression observed during adverse conditions may attenuate the growth-promoting effects of the IGFs and, thereby, contribute to the decline in fetal growth rate found in these circumstances. In contrast, the fall in the soluble form of the IGF-II type 2 receptor during fetal undernutrition may increase availability of plasma IGF-II and promote tissue differentiation, while maintaining a basal stimulus to fetal growth in the face of low IGF-1 bioavailability.

The nutritionally induced alterations in fetal IGFBP expression may be due, in part, to the concomitant changes in the fetal endocrine environment. In fetal ungulates, hepatic expression and plasma concentrations of IGFBP-1 are reduced by insulin and increased by catecholamines and thyroxine (Latimer et al., 1993; Gallaher et al., 1994; Hooper et al., 1994). Furthermore, since the ontogenic changes in IGFBP expression closely parallel the normal prepartum rise in plasma cortisol in the sheep fetus (Carr et al., 1995; Fowden, Li and Forhead, 1998), glucocorticoids may also be involved in regulating IGFBP production in utero as occurs in postnatal animals (Allan, Flint and Patel, 2001). Certainly, in human infants, antenatal glucocorticoid treatment lowers plasma IGFBP-1 and raises plasma IGFBP-3 concentrations at delivery (Kajantie et al., 2001).

The effects of the glucocorticoids on the IGF axis may provide a mechanism for the intrauterine programming of adult disease. Human epidemiological observation and experimental studies on animals have shown that impaired intrauterine development is associated with postnatal abnormalities in cardiovascular and metabolic function, which, in humans, lead to an increased incidence of adult-onset degenerative diseases, such as coronary heart disease and Type II diabetes (Barker, 2001; Bertram and Hanson, 2001). Precocious elevations in fetal plasma cortisol induced by sub-optimal conditions in utero may cause a premature transition from IGF-II to IGF-I production with beneficial effects on tissue differentiation should delivery occur before full term. However, if delivery is not stimulated prematurely, the cortisol-induced switch from the fetal to the adult mode of somatotrophic regulation may lead to inappropriate changes in cell proliferation and differentiation in utero with adverse sequelae both at birth and much later in life.

CONCLUSIONS

Both *Igf* genes have important roles in feto-placental growth but their expression and specific actions differ. Their effects can also be amplified or attenuated by the IGFBPs. Although *Igf1* gene expression is low in the fetus, IGF-1 appears to have a more prominent role than IGF-II in modulating cell proliferation in relation to the specific endocrine and nutritional conditions prevailing in utero (Figure 3). Tissue expression and circulating levels of IGF-I are regulated by the nutrient supply and enhance the uptake and utilization of substrates by the fetal tissues. This anabolic effect of IGF-1, particularly on fetal amino acid metabolism leads to tissue accretion and growth of the fetus (Figure 3). fetal IGF-I, therefore, stimulates fetal growth when nutrients are available and, thereby, ensures that the fetal growth rate is commensurate with the

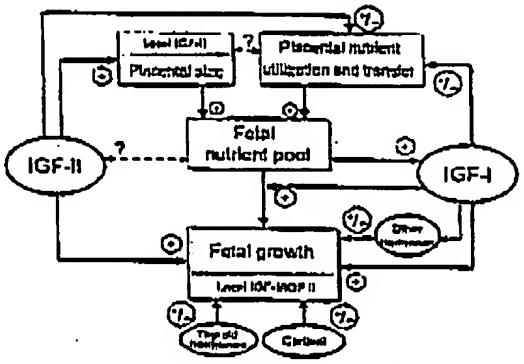

Figure 3. Schematic diagram showing the role of fetal IGF-I and IGF-II in the control of feto-placental growth. Solid line=known effects, dotted line=possible effects, + positive effects, − inhibitory effects, ○ circulating hormones, □ physiological systems.

nutrient supply. In part, the actions of IGF-I on tissue growth may be mediated by changes in other growth regulatory fetal hormones (Figure 3). fetal IGF-I has also been shown to modify placental nutrient utilization and transfer but appears to have relatively little effect on placental size (Figure 3). The *Igf2* gene, on the other hand, is highly expressed in-utero and has a major role in placental growth and nutrient transfer (Figure 3). It appears to provide the constitutive drive for intrauterine growth via both its placental effects and direct paracrine actions in the fetal tissues. Compared to *Igf1*, the *Igf2* gene is relatively unresponsive to nutritional stimuli. However, in specific fetal tissues, it does respond to changes in the fetal glucocorticoid concentration (Figure 3). fetal IGF-II may, therefore, be responsible for the developmental and tissue specific changes in cell differentiation that occur in key tissues close to term and during adverse intrauterine conditions when fetal cortisol levels are high.

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Maternal Insulin-Like Growth Factors-I and -II Act via Different Pathways to Promote Fetal Growth

Amanda N. Sferruzzi-Perri, Julie A. Owens, Kirsty G. Pringle, Jeffrey S. Robinson, and Claire T. Roberts

Research Center for Reproductive Health, Discipline of Obstetrics and Gynecology, University of Adelaide, Adelaide, South Australia 5005, Australia

The placenta transports substrates and wastes between the maternal and fetal circulations. In mice, placental IGF-II is essential for normal placental development and function but, in other mammalian species, maternal circulating IGF-II is substantial and may contribute. Maternal circulating IGFs increase in early pregnancy, and early treatment of guinea pigs with either IGF-I or IGF-II increases placental and fetal weights by mid-gestation. We now show that these effects persist to enhance placental development and fetal growth and survival near term. Pregnant guinea pigs were infused with IGF-I, IGF-II (both 1 mg/kg·d), or vehicle sc from d 20–38 of pregnancy and killed on d 62 (term ~ 69 d). IGF-II, but not IGF-I, increased the mid-sagittal area and volume of placenta devoted to exchange by approximately 30%, the total volume of trophoblast and maternal blood spaces within the placental exchange region (+20% and +40%, respectively), and the total surface area of placenta for exchange by 30%. Both IGFs reduced resorptions, and IGF-II increased the number of viable fetuses by 26%. Both IGFs increased fetal weight by 11–17% and fetal circulating amino acid concentrations. IGF-I, but not IGF-II, reduced maternal adipose depot weights by approximately 30%. In conclusion, increased maternal IGF-II abundance in early pregnancy promotes fetal growth and viability near term by increasing placental structural and functional capacity, whereas IGF-I appears to divert nutrients from the mother to the conceptus. This suggests major and complementary roles in placental and fetal growth for increased circulating IGFs in early to mid-pregnancy. (*Endocrinology* 147: 3344–3355, 2006)

THE PLACENTA IS a multifunctional organ that forms the interface between the fetal and maternal circulations. It is essential for fetal growth as it supplies the developing fetus with oxygen and nutrients, transporting them from the mother into the umbilical circulation. Abnormalities in placental structural development can impair placental function, reducing substrate supply to the fetus, and may result in intrauterine growth restriction (1). It is estimated that placental dysfunction accounts for 70–80% of growth-restricted newborns (2), currently affecting 6% of pregnancies in developed countries (3) and up to 40% in developing countries (4). Intrauterine growth restriction is associated with perinatal morbidity and mortality (5, 6) and increases the risk of poor health in childhood and adult life (7). In addition, impaired placental trophoblast invasion of the maternal uterine vasculature and/or poor placental function are implicated in other major pregnancy complications, such as miscarriage (8), preeclampsia (1), placental abruption (9), and preterm labor (10, 11). Therefore, it is imperative that we understand the factors essential for regulating placental functional development to identify causes of such diseases and as a basis for the development of therapeutics.

The IGF-I and -II have been implicated in placental structural and functional development. *Igf2* overexpression in mice causes placental and fetal overgrowth (12), whereas *Igf2* gene deletion reduces placental weight by 17% on d 13.5 and 25% on d 16.5 of gestation, with a fetal weight reduction of 40% from d 16.5 (term ~ 19 d) (13, 14). In addition, placental amino acid transporter expression is altered by *Igf2* deficiency in mice (15). Ablation of the placental-specific *Igf2* promoter (P0) in mice reduces placental weight and adversely affects placental structural differentiation and transport capacity, with reduced fetal weight evident 2 d later (16, 17). The latter reduction in fetal weight was comparable to that induced by global *Igf2* gene ablation, suggesting that the effects of *Igf2* deficiency on fetal growth are mediated by actions on the placenta in mice.

In contrast, *Igf1* gene ablation in mice does not alter placental weight but reduces fetal weight, indicating that IGF-I is important in the fetus (14, 18). IGF-I may modulate placental nutrient capacity because IGF-I administration to pregnant rats, or increased endogenous expression in pregnant mice, increases the weight of the fetus but not that of the placenta (19). IGF-I stimulates glucose and amino acid uptake in cultured human placental trophoblasts (20–22) and promotes placental nutrient uptake and metabolism when infused into fetal sheep (23–25). Moreover, exposure to IGF-I inhibits release of vasoconstrictors, such as thromboxane B2 and prostaglandin F2 α, in human term placental explants (26), which may increase placental blood flow and delivery of nutrients for the growth of the fetus.

The placenta is exposed to IGFs from multiple sources, including those produced locally and those circulating within the fetus and mother. Maternally derived IGFs may have a major influence on placental development, particularly in women and in guinea pigs where circulating IGFs are substantial (27, 28). Indeed, the IGF axis in guinea pigs is very similar to that of humans (29), whereas rats and mice do not

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Abbreviations: IGFBP, IGF binding protein.

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have circulating IGF-II postnatally. The placenta in guinea pigs is more similar to the human placenta than that of other nonprimate species being hemomonochorial, although it is labyrinthine rather than villous in structure. The guinea pig placenta is comprised of a labyrinth, which contains both fetal capillaries and maternal blood sinuses and provides the means for exchange between the two circulations and an interlobium that is comprised of syncytiotrophoblast and maternal blood sinuses, and is the site where much of the metabolic activity of the placenta is thought to occur (30). In the human placenta, exchange and endocrine functions are performed in the placental villi (31). In addition, placental trophoblast cells in guinea pigs are highly invasive and, like those in humans, engage in interstitial and endovascular invasion of the decidua. They remodel the uterine spiral arterioles to permit the large increase in blood flow to the placenta (32, 33) that is essential for placental growth and subsequent function and therefore fetal growth.

In the guinea pig, major structural determinants of placental function are strongly predicted by maternal IGF-II concentration in mid-pregnancy and by maternal IGF-I in late pregnancy (34, 35). Furthermore, in this species, food restriction reduces maternal plasma IGF concentrations (36) that correlate with delayed structural and functional maturation of the placenta and with reduced fetal growth (34, 35, 37). The structural defects in the placenta of food-restricted guinea pigs are similar to those seen in placentas from women with preeclampsia (34). In addition, reduced maternal plasma IGF-I in pregnant women is associated with placental dysfunction and small-for-gestational-age (38, 39) or growth-restricted infants (40).

Consistent with these adaptive changes in maternal IGFs regulating placental development, maternal supplementation with IGF-I or IGF-II in early to mid-pregnancy in the guinea pig increases placental and fetal weights by mid gestation (41). Therefore, we suggest that the increased maternal production of both IGFs in early pregnancy is an important adaptation to pregnancy, which promotes placental functional development and consequently fetal growth. Whether anabolic effects of an increased abundance of maternal IGFs in early pregnancy on the placenta would persist into late gestation and affect the fetus is currently unknown. Therefore, the aim of this study was to determine the effects of maternal IGF-I and -II supplementation in early to mid-pregnancy on placental development and fetal growth and viability near term.

Materials and Methods

Animals

This study was approved by the University of Adelaide, Animal Ethics Committee. Virgin guinea pigs (IMVS colored strain, approximately 500 g, 3–4 months old) were housed individually in the University of Adelaide Medical School Animal House. Guinea pigs were provided with food and water *ad libitum*. Females were examined daily for estrus indicated by a ruptured vaginal membrane (complete estrous cycle lasts approximately 15 d) and mated naturally with a male. The day a copulatory plug was observed was designated as d 1 of pregnancy. From 2 wk before mating, body weight was monitored three times weekly. Females were assigned to three groups of similar mean weight at mating.

On d 20 of pregnancy (term 69–70 d), females were anesthetized with atropine sulfate (0.05 mg/kg, sc; Apex Laboratories, Sydney, Australia), xylazine hydrochloride (4 mg/kg, im; Troy Laboratories, Sydney, Australia), ketamine hydrochloride (25 mg/kg, ip; Troy Laboratories) and administered local analgesia with lignocaine hydrochloride (Troy Laboratories). A 200-μl mini osmotic pump (Alzet 2002; Alzet, San Francisco, CA) was surgically inserted sc. Minipumps had previously been prepared to deliver vehicle (0.1 M acetic acid) (n = 7) or 1 mg/kg·d IGF-II (n = 7) or IGF-I (n = 9) (human recombinant protein; GroPep Pty. Ltd., Adelaide, Australia) for 18 d at a flow rate of 0.51 μl/h.

On d 62 of pregnancy, guinea pigs were killed by overdose of sodium pentobarbitone (Lethobarb; Virbac, Sydney, Australia). Viable and resorbing implantation sites were counted and the uterus and its contents, viable fetuses, and placentae were weighed. Fetal biparietal diameter, abdominal circumference, and crown-to-rump length were measured. A 3-mm mid-sagittal placental slice was fixed in 4% paraformaldehyde for structural analysis. Analyses of body composition were performed on the mothers and all fetuses to determine the absolute and relative weights of adrenals, kidneys, pancreas, liver, spleen, heart, brain, lungs, gastrointestinal tract, reproductive tract, biceps, triceps, gastrocnemius and soleus muscles and retroperitoneal, perirenal, and interscapular adipose tissues. Skin and carcass weights of the dams and carcass weight of the fetuses were also recorded.

Measurement of maternal circulating IGF-I, IGF-II, and IGF binding proteins (IGFBPs)

In an additional cohort of guinea pigs (vehicle, n = 5; IGF-I, n = 5; IGF-II, n = 3), mothers were killed on d 35 of pregnancy, while the minipumps were still active by overdose of sodium pentobarbitone. Maternal blood was collected by cardiac puncture and centrifuged at 2500 rpm for 15 min at 4 C, then plasma was recovered and stored at −20 C.

Plasma IGF-I and IGF-II proteins were dissociated from their binding proteins (IGFBPs) by size exclusion high pressure liquid chromatography performed at pH 2.5, as previously described (42, 43). From each acidified plasma sample, four fractions were eluted from the column, and fraction 1, which contained only IGFBPs, and fraction 3, which contained only the IGFs, were collected for later analysis. The IGF fraction 3 was analyzed by specific RIAs for IGF-I and IGF-II concentrations as previously described (42, 44).

Recombinant human IGF-I and IGF-II (GroPep Pty. Ltd.) were used as standards and for preparation of radiolabeled ligands. IGF-I was measured by RIA using rabbit antihuman IGF-I (MAC Ab 89/1; GroPep Pty. Ltd.) at a final dilution of 1/60,000 and a monoclonal mouse antirat IGF-II antibody (kind gift from Dr. K. Nishikawa, Kanaza Medical University, Ishikawa, Japan) was used at a final concentration of 1/500 to measure IGF-II by RIA. Cross-reactivity of IGF-II in the IGF-I RIA was less than 1% (44) and that of IGF-I in the IGF-II RIA was less than 2.5% (45). Both IGF-I and IGF-II amino acid sequences are remarkably conserved across species. Guinea pig IGF-I and IGF-II have previously been shown to have 100% amino acid sequence identity to those of human (46, 47), whereas guinea pig IGF-II has only one amino acid different to that of the rat (48). We have previously reported that the recoveries of IGF-I and IGF-II are more than 95% for these assays (28). The minimal detectable concentrations of IGF-I and IGF-II were 6.61 and 9.48 ng/ml, respectively. The samples were analyzed in a single RIA, where the mean intraassay coefficients of variation were 3.7 and 5.6% for IGF-I and IGF-II RIAs, respectively.

The total IGFBP binding capacity in the maternal circulation was indirectly measured as the interference of the IGFBPs in fraction 1 in the IGF-I RIA, as previously described (42). The ratio of IGFs to IGFBPs provided an index of IGF bioavailability in the maternal circulation.

Placental histology

Mid-sagittal slices of placentae that had been fixed in 4% paraformaldehyde overnight were washed in 1% PBS, dehydrated, and embedded in paraffin wax, then 5-μm sections were stained with Masson's Trichrome (49). From each dam, one to three placentae were randomly selected for histological assessment. The cross-sectional areas of the placental interlobium (germinative region) and labyrinth (exchange region) were measured in complete mid-sagittal sections using an Olympus BH-2 microscope with ×2 objective and ×3.3 ocular lenses and video

Image analysis software (Video Pro; Leading Edge, Adelaide, Australia). The proportion (percentage) of each region in the placenta was then estimated by dividing the cross-sectional area of that region by the total mid-sagittal cross-sectional area of the placenta. An estimate of the volume of these regions was then calculated by multiplying their proportion by total placental weight.

Structure of the placental exchange region (labyrinth)

To distinguish cell types within the placental labyrinth, mid-sagittal sections of placenta were double-labeled with mouse antibodies to human vimentin (3B4; Dako, Glostrup, Denmark) and human pan cytokeratin (C2562; Sigma, Sydney, Australia) to identify fetal capillaries and trophoblast, respectively, and then stained with eosin to aid the identification of maternal blood spaces. This employed a triple layer technique for each antibody, performed sequentially. Sections were deparaffinized and brought to water. For antigen retrieval, sections were incubated at 37 C for 15 min in 0.03% protease (Sigma). Endogenous peroxidase activity was quenched by incubating with 3% hydrogen peroxide in water for 30 min. Sections were then washed in three changes of PBS for 5 min each and blocked for nonspecific binding with serum-free protein block (Dako) for 10 min without washing. 3B4 antibody diluted 1:50 with 10% normal guinea pig serum and 1% BSA was applied first and incubated overnight in a humidified chamber at room temperature. Sections were washed as above, and biotinylated goat anti-mouse IgG secondary antibody (Dako) was applied for 30 min, followed by washing. Streptavidin conjugated to horseradish-peroxidase (Rockland Immunochemicals, Pottstown, PA) was applied for 10 min, then sections were washed as above. 3B4 binding was visualized by incubating with diaminobenzidine with 2% ammonium nickel (II) sulfate (Sigma) to form a black precipitate. The process was then repeated for the second primary antibody (C2562) diluted 1:50 with PBS, 10% normal guinea pig serum, and 1% BSA, but nickel was omitted from the chromogen, leaving a brown precipitate. Negative controls used irrelevant mouse IgG in place of the primary antibodies or the primary antibody diluent on its own.

The placental labyrinth was then morphometrically analyzed, as previously described (34). Briefly, the proportions (volume density) and volumes of the labyrinthine placental components were quantitated by point counting on 10 nonoverlapping fields with random systematic sampling using an Olympus BH-2 microscope with ×20 objective and ×3.3 ocular lenses. The weight of each component was estimated by multiplying the volume density by the weight of the placental labyrinth. The surface area per gram of placental labyrinth was quantitated using intercept counting and the total surface area of syncytiotrophoblast for exchange and arithmetic mean trophoblast thickness (the layer through which substrate exchange occurs) were calculated as previously described (34).

Protein localization of IGF receptors in the placenta on d 35 of pregnancy

To determine that the placenta expressed the type 1 and 2 IGF receptors at the time of treatment we localized them in placental sections from the cohort of guinea pigs that were killed on d 35 of pregnancy in which circulating IGFs had been quantified. Mid-sagittal slices of placentae were immuno-labeled with rabbit antibodies raised against human IGF1R (N-20, diluted 1:20; Santa Cruz Biotechnology, Santa Cruz, CA) and IGF2R (a kind gift from Dr. Carolyn Scott, Kolling Institute of Medical Research, Sydney, Australia; diluted 1:100). This employed a triple layer technique for each antibody performed on serial placental sections, as described above. Negative controls used irrelevant mouse IgG in place of the primary antibodies or the primary antibody diluent on its own.

Plasma metabolite and hormone concentrations

Maternal and fetal plasma glucose (glucose HK assay kit; Roche Diagnostics, Mannheim, Germany), free-fatty acids (WAKO Nefa C free fatty acid kit; NovoChem, Nieuwegein, The Netherlands), cholesterol (cholesterol CHOD-PAP assay kit; Roche Diagnostics), and triglycerides (triglycerides assay kit; Roche Diagnostics) were quantified with enzymatic assay kits using a COBAS Mira automated centrifugal analyzer (Roche Diagnostics). Maternal and fetal plasma α-amino nitrogen concentrations were determined using the β-naphthaquinone sulfonate colorimetric assay as previously described (50). Maternal plasma estradiol (Ultra-Sensitive Estradiol; Diagnostic Systems Laboratories, Houston, TX) and progesterone concentrations (progesterone assay kit; Diagnostic Systems Laboratories) were quantified with RIA kits.

Statistics

To assess differences in fetal weight distribution between treatments, χ^2 tests were performed using Microsoft Excel. All other data were analyzed using SPSS version 13 (SPSS, Chicago, IL). To assess differences in maternal weight gain, repeated measured ANOVA with Bonferroni *post hoc* tests were performed. To assess differences in maternal body composition, general linear model univariate ANOVA with Bonferroni *post hoc* tests were performed. To assess differences in fetal and placental parameters, linear mixed model repeated measures ANOVA with Bonferroni *post hoc* tests were performed with the mother as a subject and the fetus or placenta as the repeated measure. The number of viable pups per litter were used as a covariate when required. Data are expressed as mean ± SEM or estimated marginal mean ± SEM as required. Data were considered statistically significant when $P < 0.05$.

Results

Exogenous maternal IGF treatment increases maternal plasma IGF-I and IGF-II

To determine the concentration of IGFs we achieved in the maternal circulation in response to this treatment, an additional cohort of guinea pigs was killed on d 35 of pregnancy, while the minipumps were still active. Exogenous IGF-I increased maternal plasma IGF-I by 340% ($P = 0.001$) and reduced that of IGF-II by 45% ($P = 0.008$; Fig. 1). Exogenous IGF-II did not alter plasma IGF-I concentrations but increased plasma IGF-II by 240% ($P = 0.004$; Fig. 1). In addition, the total apparent IGFBP activity in maternal plasma was not altered by exogenous IGF. Maternal IGF-I treatment increased the ratio of IGF-I to IGFBPs in plasma by 230% ($P = 0.004$), whereas IGF-II increased the ratio of IGF-II to IGFBPs in plasma by 125% ($P = 0.04$; Fig. 1).

IGF receptor proteins are expressed by the guinea pig placenta during the treatment

To establish that IGF1R and IGF2R are expressed by the guinea pig placenta during the IGF treatment, immunolabeling was performed on guinea pig placenta recovered from vehicle-treated mothers killed on d 35 of pregnancy (Fig. 2). IGF1R and IGF2R were ubiquitously expressed by the guinea pig placenta, with profuse cytoplasmic staining observed in trophoblast and fetal endothelium of the labyrinth and trophoblast of the interlobium (Fig. 2, A and C). Both IGF receptor proteins were concentrated on the apical surface of trophoblast within large maternal blood sinusoids and within maternal blood spaces (Fig. 2, B and D).

Exogenous maternal IGF-II, but not IGF-I, enhances development of the placental exchange region (labyrinth)

IGF treatment in early to mid-pregnancy did not alter placental weight in late gestation (Table 1). However, there was a 17% difference in placental weight between IGF-I- and IGF-II-treated mothers ($P = 0.039$). Exogenous IGF-II increased placental labyrinthine cross-sectional area by 28% ($P = 0.005$) but not that of the interlobium (Fig. 3, A–C, and

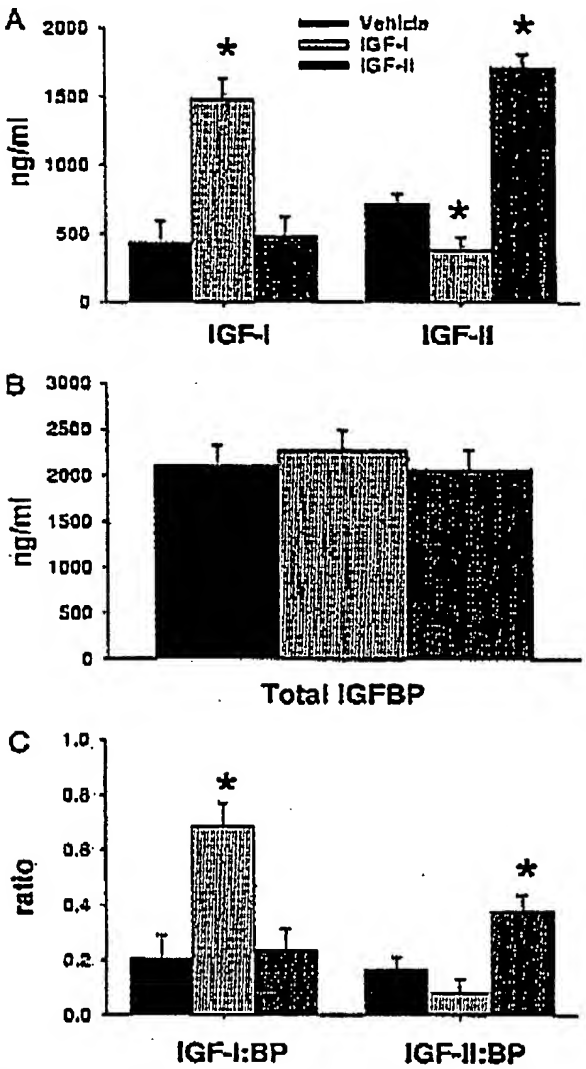

FIG. 1. The effect of exogenous maternal IGFs on maternal circulating IGF-I, IGF-II (A), and total IGFBP (B) concentrations and bioavailability of IGFs in the circulation indicated by IGF to IGFBP ratios (C) during treatment on d 35 of pregnancy. Data are from three to six mothers per treatment, and values are expressed as means ± SEM. *Asterisks* denote a statistically significant difference compared with the vehicle group, $P < 0.034$.

Table 1). The ratio of labyrinth to interlobium was increased by IGF-II (+37%, $P = 0.054$). IGF-II increased the proportion of the placenta comprised of labyrinth (+9%, $P = 0.0003$) and reduced that composed of the interlobium (−24%, $P = 0.0003$) (Table 1). IGF-II also increased the volume of placental labyrinth (+28%, $P = 0.027$) but did not alter that of the interlobium (Table 1). Maternal IGF-I treatment did not alter any placental parameter (Table 1).

To examine placental labyrinthine development in response to earlier maternal IGFs in more detail, structural correlates of placental function were quantified. Maternal IGF treatment did not alter the proportions of the placental labyrinth composed of trophoblast, maternal blood spaces, or fetal capillaries (Fig. 4A). IGF-II increased the volume of trophoblast (+29%, $P = 0.015$) and that of maternal blood spaces (+46%, $P = 0.035$) within the placental labyrinth (Fig. 4B). The total surface area of trophoblast functioning in exchange was also increased by IGF-II (+39%, $P = 0.037$, Fig. 4C). There was no effect of IGF treatment on syncytiotrophoblast barrier thickness (vehicle, 4.7 ± 0.2 μm; IGF-I, 4.8 ± 0.2 μm; IGF-II, 4.4 ± 0.2 μm). Maternal IGF-I treatment did not affect any placental labyrinthine structural parameter measured.

Exogenous maternal IGFs increase fetal survival

Maternal IGF treatment did not affect total litter size (Table 2). However, the number of resorptions was reduced by IGF-I (−77%, $P = 0.009$) and IGF-II (−60%, $P = 0.01$), while IGF-II also increased the number of viable fetuses (+25%, $P = 0.034$) near term (Table 2). Maternal IGFs did not alter the ratio of females to males (Table 2).

Exogenous maternal IGFs increase fetal growth with IGF-specific effects on fetal body composition

Maternal IGF-I and IGF-II treatment in early to mid-pregnancy increased fetal weight near term by 17% ($P = 0.002$) and 11% ($P = 0.042$), respectively (Table 3). Both maternal IGF treatments significantly skewed the fetal weight distribution to the right (both $P < 0.0005$; Fig. 5A). The percentage of fetuses heavier than 81 g was 5% in controls, 37% in IGF-I, and 19% in IGF-II-treated animals (Fig. 5A). IGF-I treatment increased fetal crown-to-rump length by 9% ($P = 0.014$), as well as abdominal circumference by 10% ($P = 0.05$). IGF-I increased the fetal weight to placental weight ratio by 29% (vehicle, 14.82 ± 0.86; IGF-I, 19.14 ± 0.73; IGF-II, 16.18 ± 0.65; $P < 0.01$). Fetal weight correlated positively with placental weight across all treatments ($r = 0.27$, $P = 0.026$) and within each of the IGF-I and IGF-II treatment groups ($r = 0.44$, $P = 0.042$ and $r = 0.40$, $P = 0.038$, respectively) but not in vehicle-treated dams alone (Fig. 5B). Overall, fetal weight correlated positively with both the mid-sagittal cross-sectional area and the estimated total volume of the placental labyrinth ($r = 0.58$, $P = 0.009$ and $r = 0.43$, $P = 0.006$, respectively), as well as the volume of trophoblast and fetal capillaries in the placental labyrinth ($r = 0.34$, $P = 0.034$ and $r = 0.62$, $P < 0.001$, respectively).

Maternal IGF-I treatment increased fetal carcass weight (+19%, $P = 0.002$), increased the combined weights of fetal kidneys (+20%, $P = 0.028$), caecum (+24%, $P = 0.027$), total gastrointestinal tract (+13.5%, $P = 0.049$), and the combined fetal fat depots (+16%, $P = 0.028$) (Table 3). Conversely, IGF-I reduced the fractional weights of the fetal spleen (−21%, $P = 0.001$), liver (−12.5%, $P = 0.002$), and brain (−18.5%, $P = 0.004$) (Table 3). Both IGF-I and IGF-II increased the weights of the fetal retroperitoneal fat (+24%, $P = 0.004$; +18%, $P = 0.031$, respectively) and combined fetal muscle mass (+22%, $P = 0.008$; +19%, $P = 0.024$, respectively; Table 3). IGF-I and IGF-II also increased the fetal triceps absolute (+29%, $P = 0.001$; +24%, $P = 0.01$, respectively) and relative weights (both +16%, $P < 0.03$, Table 3). Body composition of male

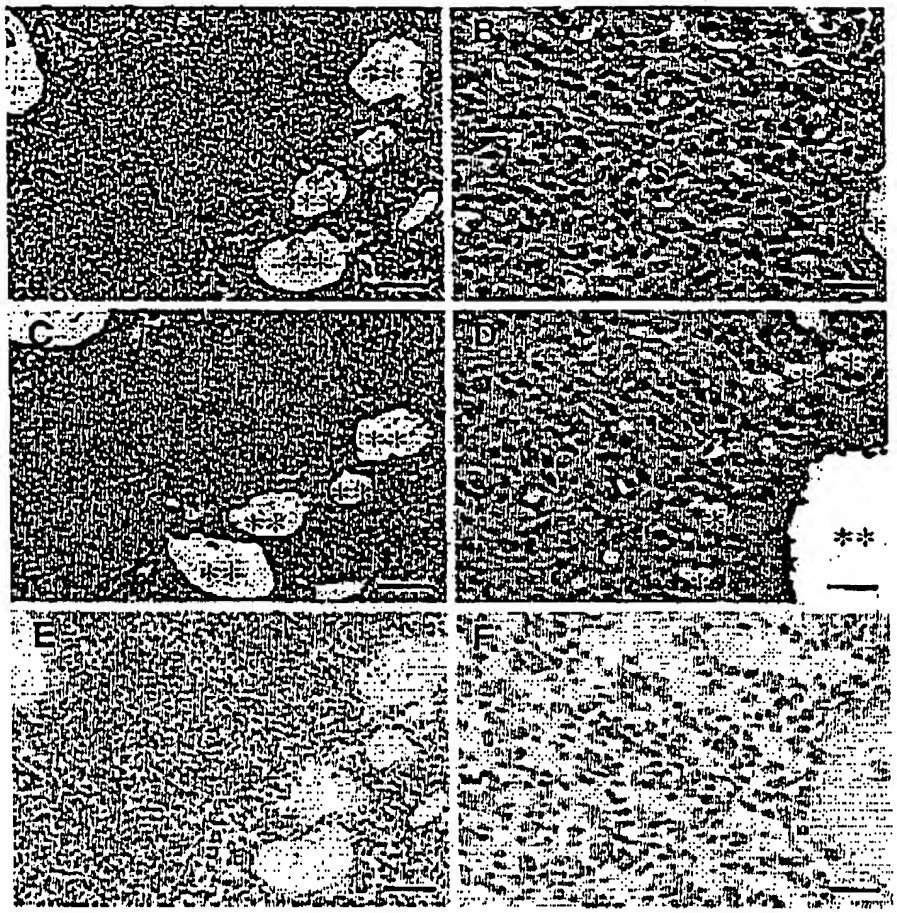

FIG. 2. Representative mid-sagittal serial sections of placentae on d 35 of pregnancy immunolabeled for the type 1 (A and B) and type 2 (C and D) IGF receptors. Representative negative control placental sections displayed (E and F). *Two asterisks* indicate maternal blood sinusoids and *single asterisks* indicate maternal blood spaces. *Scale bars*, 400 μm (A, C, and E) and 40 μm (B, D, and F).

and female fetuses was similar and was similarly affected by maternal IGF treatment (data not shown).

Exogenous maternal IGFs increase concentrations of amino acids in the fetal circulation

Maternal IGF-I and IGF-II treatment increased fetal circulating amino acid concentrations (+196%, $P = 0.026$ and +137%, $P = 0.029$, respectively) and maternal IGF-I reduced fetal circulating cholesterol concentrations (−30%, $P = 0.049$) near term (Fig. 6A). There was no effect of treatment on fetal plasma glucose, triglyceride, or free fatty acid concentrations (Fig. 6A).

Exogenous maternal IGF-I, but not IGF-II, alters maternal body composition

Weight gain and body composition analyses were performed to determine whether exogenous IGFs affected the mother. Both exogenous maternal IGF-I and IGF-II did not alter maternal weight gain during or after IGF treatment (Fig. 7), nor total body and lean body mass near term (Table 4). IGF-I reduced maternal interscapular fat depot weight (−25%, $P = 0.028$) and the fractional weights of the perirenal (−32%, $P = 0.05$), retroperitoneal (−33%, $P = 0.037$), and interscapular fat (−28%, $P = 0.01$; Table 4). IGF-I reduced the absolute and fractional weights of the combined adipose depot weights in the mother by approximately 30%, ($P = 0.016$ and $P = 0.007$, respectively). IGF-II did not alter the absolute or relative weights of any maternal organ or tissue examined.

Exogenous maternal IGF treatment does not alter maternal circulating metabolite concentrations

Maternal IGF treatment did not alter circulating concentrations of glucose, free fatty acids, amino acids, triglycerides, or cholesterol in the mother near term (Fig. 6B).

Exogenous maternal IGF treatment and maternal circulating hormone concentrations

To determine whether treatment of the mother during early to mid-pregnancy with IGFs altered maternal circulating estradiol (Fig. 7C) and progesterone (Fig. 7D), their concentrations were determined on d 35 of pregnancy in the additional cohort of guinea pigs in which the plasma IGF and IGFBP concentrations were determined as described above. Treating the mother during early to mid-pregnancy with IGF-I doubled circulating maternal estradiol concentrations

TABLE 1. Effect of maternal IGF treatment on placental structure near term

	Vehicle	IGF-I	IGF-II
Placental weight (g)	4.63 ± 0.29^{a,b}	4.11 ± 0.24^{a}	4.84 ± 0.22^{b}
Cross-sectional area labyrinth (mm^2)	98.9 ± 3.8^{a}	112.3 ± 8.0^{a}	126.6 ± 8.3^{b}
Cross-sectional area interlobium (mm^2)	35.6 ± 2.8	32.6 ± 4.3	30.4 ± 2.6
Labyrinth:interlobium	3.10 ± 0.43	3.89 ± 0.44	4.23 ± 0.35
Proportion labyrinth (%)	73.6 ± 1.2^{a}	77.6 ± 1.1^{a,b}	80.5 ± 1.1^{b}
Proportion interlobium (%)	26.4 ± 1.2^{a}	22.4 ± 1.1^{a,b}	19.5 ± 1.1^{b}
Volume labyrinth (cm^3)	3.34 ± 0.25^{a}	3.20 ± 0.23^{a}	4.26 ± 0.23^{b}
Volume interlobium (cm^3)	1.21 ± 0.09	0.95 ± 0.09	1.03 ± 0.08

Data are expressed as mean ± SEM from seven to nine dams per treatment with one to three placentae randomly selected for histological analysis.

Different superscripts denote differences between groups, *a* vs. *b*, $P < 0.039$.

in late pregnancy, although this was not quite significant ($P = 0.078$). IGF-I treatment did not alter mid or late pregnancy circulating progesterone concentrations. Exogenous maternal IGF-II during early to mid-pregnancy increased circulating estradiol concentrations (+150%) in mid-pregnancy and progesterone concentrations in mid (+53%) and late (+83%) pregnancy in the mother; however, these also did not reach statistical significance ($P > 0.08$) (Fig. 7, C and D).

Discussion

The present study demonstrates for the first time that administration of IGF-II to the mother in early to mid-pregnancy increases placental structural and functional capacity by increasing the volume and surface area of the exchange region of the placenta near term, whereas IGF-I has no effect on the placenta. IGF-I, in contrast, reduced maternal adiposity late in pregnancy, whereas IGF-II did not affect maternal body composition. Importantly, however, maternal treatment with either IGF in early to mid-pregnancy substantially reduced fetal resorptions, increased fetal weight, and increased fetal circulating amino acid concentrations near term. Furthermore, administration of IGF-II also increased fetal viability in late pregnancy. This suggests that maternal IGF abundance, particularly that of IGF-II, during the period of early placental growth and development may determine in part the margin of safety between placental capacity to deliver, and fetal demand for, substrates throughout pregnancy.

Specifically, in the current study, administration of 1 mg/kg·d IGFs increased the abundance of maternal circulating IGF-II and IGF-I by 2.5- to 3.4-fold, during early to mid-pregnancy. The concentration of free IGF to IGFBP ratio in the maternal plasma, and hence bioavailable IGF, was also substantially increased. Similar IGF treatment of guinea pigs during early to mid-pregnancy increased placental weight at mid-gestation (41), which was not sustained to near term in the current study. Importantly, however, the functional capacity of the placenta, as indicated by the mid-sagittal cross-sectional area, proportion and volume of the region devoted to exchange (labyrinth) were increased late in gestation, by prior maternal IGF-II treatment. Furthermore, although the composition of this exchange region of the placenta was unaltered by earlier maternal IGF treatment, the total volume of trophoblast and maternal blood spaces, as well as the total surface area of placenta functioning in exchange were increased by IGF-II. As the labyrinth expands at the expense of the interlobium in the second half of pregnancy in the guinea

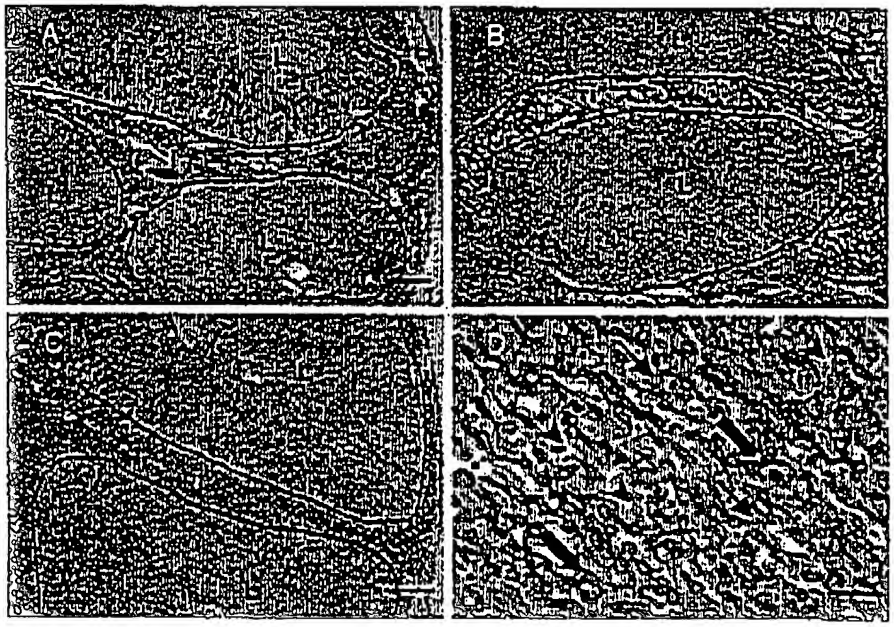

FIG. 3. The effect of exogenous maternal IGF treatment on placental structure. Representative mid-sagittal sections of near-term placentae stained with Masson's Trichrome to distinguish labyrinth and interlobium layers from mothers that had been treated with vehicle (A), IGF-I (B), or IGF-II (C) during early to mid-pregnancy. L, Labyrinth; I, interlobium. *Scale bars*, 400 μm. D, Representative mid-sagittal section of near-term placenta double-labeled and eosin stained to reveal structural components of the placental labyrinth, including fetal trophoblast (*thin arrow*), maternal blood spaces (*asterisks*), and fetal capillaries (*broad arrows*). *Scale bar*, 40 μm.

pig (30, 34, 51), together these changes in the structure of the placenta as a result of earlier exogenous maternal IGF-II are suggestive of a more mature placenta and would be expected to increase placental transport capacity. In contrast, maternal exogenous IGF-I had no effect on placental structural development.

Rapid placental structural differentiation and growth occurs in early to mid gestation in all eutherian mammals. In humans and guinea pigs, trophoblasts invade deep within the uterus and its arterioles, extensively remodeling them, to permit increased maternal blood flow to the placenta (32, 52, 53). This ensures delivery of oxygen and nutrients to the placenta, and subsequently to the fetus. The sustained effects of maternal IGF-II supplementation in early to mid-pregnancy on the placenta reported here are the converse of those observed after specific deletion of IGF-II within the placenta. IGF-II is abundantly expressed by invasive trophoblasts of human (54), mouse (55), rat (56), and guinea pig placenta (57). Ablation of placenta-specific *Igf2* gene expression (P0 transcript) in mice reduced the surface area for exchange, increased the exchange barrier thickness and also impaired nutrient transport capacity of the placenta (16, 17).

Reduced maternal circulating IGF-II in mid-pregnancy, as a result of undernutrition in guinea pigs (36), is associated with similar consequences to those of placental *Igf2* gene deletion (17), with a delay and impairment in the functional maturation of the placenta and with reduced fetal growth in both mid and late gestation (37). Together these findings indicate that maternal circulating IGF-II may act in an endocrine fashion to modulate placental development, in addition to any autocrine/paracrine effects of locally produced IGF-II. We suggest that exposure to increased circulating maternal IGF-II in early to mid-pregnancy may provide a foundation of increased placental trophoblast proliferation and invasion of the uterus and its vasculature, which leads to increased volumes of both trophoblast and maternal blood spaces in the placental labyrinth in late gestation. This would be expected to increase maternal blood flow to the placenta and enhance growth of the area devoted to exchange improving placental transfer of oxygen and nutrients to the fetus from the mother. This was consistent with increased circulating fetal amino acid concentrations with earlier maternal IGF treatment, near term. Hence, maternal IGF-II supplementation presumably increased fetal growth and viability predominantly by these actions on the placenta. Current studies in our laboratory are focused on determining whether early maternal IGF treatment increases placental transport of nonmetabolizable analogs of glucose and amino acids in the fetal circulation and tissues and whether treatment affects nutrient partitioning in the mother.

Supplementing the mother during early to mid-pregnancy with either IGF had a sustained positive effect on fetal weight, length, and girth near term, which is consistent with the anabolic effects on the fetus seen at mid-pregnancy after similar treatment in the guinea pig (41). The increased fetal weight observed with maternal IGF treatment appears to be substantially due to increased muscle mass overall and proportionately for selected muscles and perhaps enhanced fetal bone growth as indicated by increased carcass weights. This may be metabolically beneficial in later life because muscle

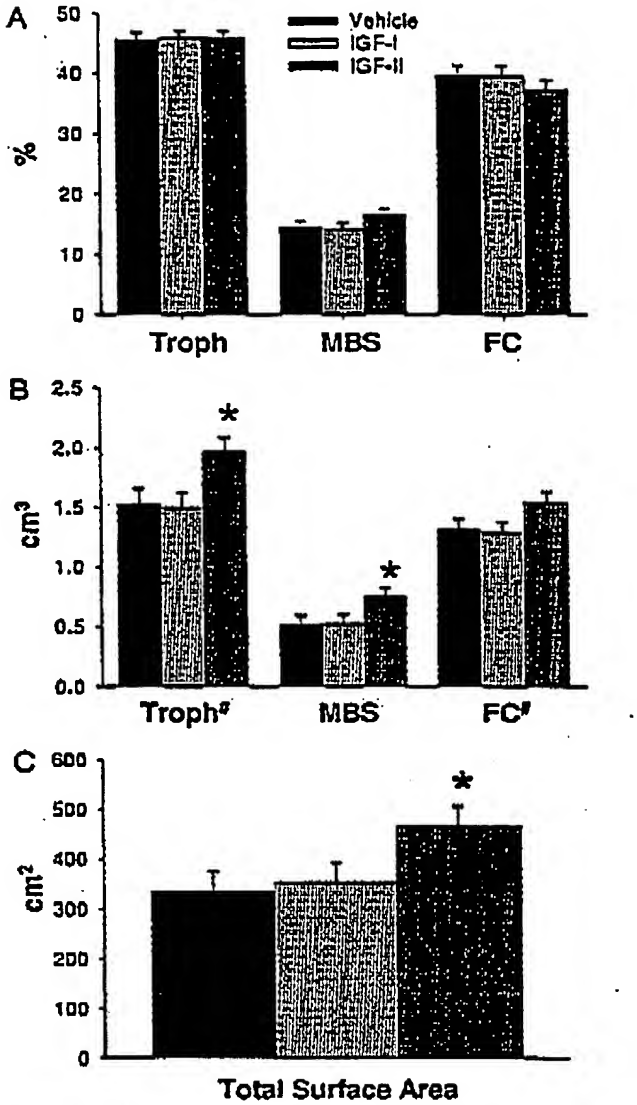

FIG. 4. The effect of exogenous maternal IGFs on structural correlates of placental exchange function near term. Proportions (A) and volumes (B) of fetal trophoblast, maternal blood spaces, and fetal capillaries in the placental labyrinth (exchange region), as well as the total surface area of syncytiotrophoblast for exchange (C). Data are from $n = 1$–3 placentae from each of seven to nine mothers per treatment. Values are expressed as means ± SEM. *Asterisks* denote a statistically significant difference compared with the vehicle group, $P < 0.05$. #, Positive correlation with fetal weight, $r > 0.34$ and $P < 0.034$.

is an important site for insulin-induced glucose uptake. Indeed, fetal growth restriction in the guinea pig, induced by maternal food restriction and accompanied by reductions in circulating maternal IGF concentrations (36), is characterized by deficits in muscle mass, increased adiposity in the fetus near term (58) and with increased blood pressure and impaired glucose and cholesterol homeostasis in adult offspring (59–61).

TABLE 2. Effect of maternal IGF treatment on litter composition and fetal dimensions near term

	Vehicle	IGF-I	IGF-II
Dams	7	7	9
Fetuses	19	22	30
Females/males	9/10	12/10	14/16
Total litter	3.42 ± 0.1	3.36 ± 0.1	3.67 ± 0.1
Number viable	2.73 ± 0.3[a]	3.27 ± 0.9[a,b]	3.40 ± 0.2[b]
Number resorbing	0.68 ± 0.1[a]	0.09 ± 0.1[b]	0.27 ± 0.1[b]

Data are expressed as mean ± SEM.
Different superscripts denote significant differences between groups, $P < 0.05$.

The present study suggests that increased maternal IGF-I and IGF-II abundances during early to mid-pregnancy promote fetal growth and viability near term by multiple mechanisms. In addition to direct effects of IGF-II on placental structural development, which in the current study were positively associated with fetal weights, the IGFs may increase nutrient transporter expression (20–22) and/or placental vasodilation (26), which would allow for more substrate to be delivered to the fetus for its growth. The IGFs may also influence placental metabolism and function, which, in turn, may drive major physiological adaptations to pregnancy in the mother, including the development of insulin resistance to divert nutrients to the conceptus (62–64). This has been attributed to placental production of hormones including estrogen, progesterone, and placental lactogen (64, 65) that reduce maternal insulin secretion (64, 66) and antagonize the effects of insulin on maternal tissues, including fat deposition (65). Treatment of the mother with IGF-II enhanced placental weight in mid-pregnancy (41) and is accompanied by elevated maternal circulating estradiol and progesterone concentrations, although these were not significant. This would be expected to amplify insulin resistance and other adaptations such as fat deposition in the mother. Consistent with this, exogenous IGF-II during early to mid-pregnancy in guinea pigs increased maternal interscapular

TABLE 3. Effect of maternal IGF treatment on fetal weight and body composition near term

	Vehicle	IGF-I	IGF-II
Fetal weight (g)	66.62 ± 2.40[a]	77.75 ± 1.96[b]	74.03 ± 1.69[b]
Crown-rump length (cm)	14.00 ± 0.34[a]	15.28 ± 0.28[b]	14.77 ± 0.24[a,b]
Abdominal circumference (cm)	8.82 ± 0.28[a]	9.69 ± 0.23[b]	9.01 ± 0.20[a,b]
Head width (cm)	6.81 ± 0.46	7.07 ± 0.39	7.20 ± 0.37
Kidneys (g)	0.59 ± 0.04[a]	0.71 ± 0.03[b]	0.67 ± 0.03[a,b]
(% Body weight)	0.89 ± 0.04	0.92 ± 0.03	0.91 ± 0.03
Spleen (g)	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
(% Body weight)	0.17 ± 0.01[a]	0.13 ± 0.01[b]	0.15 ± 0.01[a,b]
Liver (g)	3.71 ± 0.18	3.77 ± 0.14	3.84 ± 0.13
(% Body weight)	5.6 ± 0.2[a]	4.9 ± 0.1[b]	5.2 ± 0.1[a]
Brain (g)	2.49 ± .07	2.51 ± 0.06	2.52 ± 0.05
(% Body weight)	3.8 ± 0.2[a]	3.1 ± 0.1[b]	3.5 ± 0.1[a,b]
Total GI tract (g)	3.33 ± .014[a]	3.78 ± 0.11[b]	3.60 ± 0.10[a,b]
(% Body weight)	5.0 ± 0.1	4.9 ± 0.1	4.9 ± 0.1
Caecum (g)	0.37 ± 0.03[a]	0.46 ± .02[b]	0.40 ± 0.02[a,b]
(% Body weight)	0.56 ± 0.03	0.59 ± 0.02	0.54 ± 0.02
Total muscle (g)	0.30 ± 0.21[a]	0.44 ± 0.16[b]	0.43 ± 0.15[b]
(% Body weight)	0.50 ± 0.02	0.57 ± 0.02	0.55 ± 0.01
Triceps (g)	0.17 ± 0.01[a]	0.22 ± 0.01[b]	0.21 ± 0.01[b]
(% Body weight)	0.25 ± 0.01[a]	0.29 ± 0.008[b]	0.29 ± 0.007[b]
Total fat (g)	2.39 ± 0.11[a]	2.77 ± 0.09[b]	2.72 ± 0.08[a,b]
(% Body weight)	3.6 ± 0.1	3.6 ± 0.1	3.7 ± 0.09
Retroperitoneal fat (g)	0.63 ± 0.04[a]	0.78 ± 0.03[b]	0.74 ± 0.03[b]
(% Body weight)	0.9 ± 0.04	1.0 ± 0.03	1.0 ± 0.03
Carcass (g)	48.68 ± 2.0[a]	58.01 ± 1.6[b]	53.08 ± 1.5[a,b]
(% Body weight)	73 ± 0.8	75 ± 0.6	74 ± 0.6

Data expressed as estimated marginal means ± SEM adjusted for the number of viable fetuses per litter. Only tissues that were significantly affected by treatment are shown. *Different superscripts* denote significant differences between groups, *a vs. b*, $P < 0.05$.

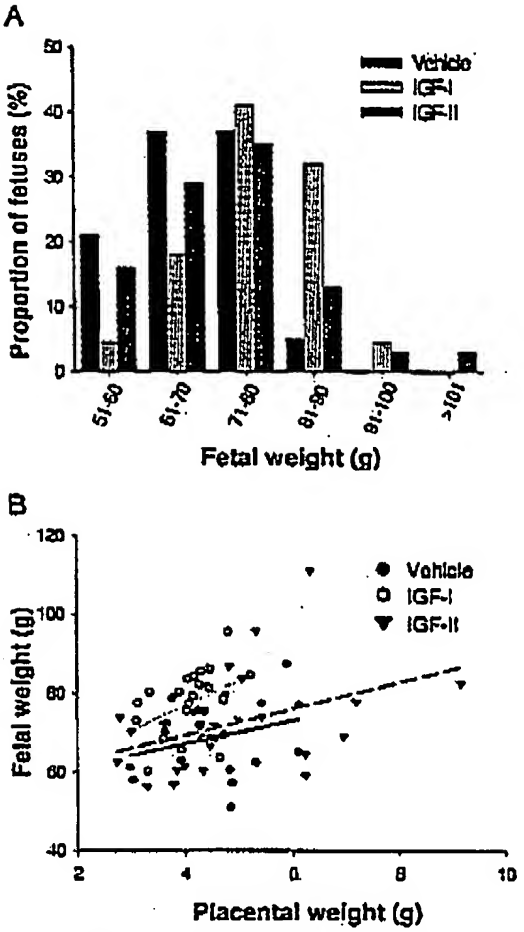

FIG. 5. The effect of exogenous maternal IGF treatment on fetal weight distribution (A) and on the association of fetal weights with placental weights (B). Each fetus from seven to nine mothers per treatment is represented.

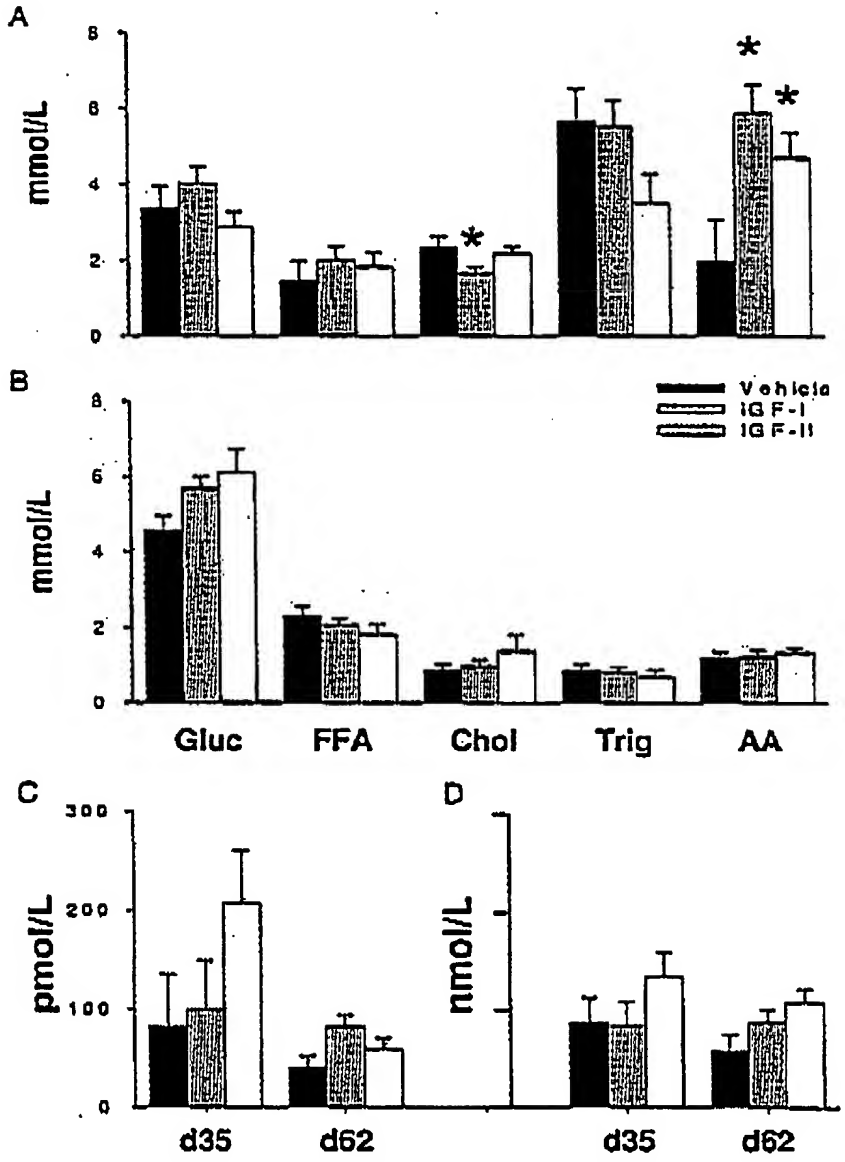

FIG. 6. The effect of exogenous maternal IGFs on circulating metabolites in the fetus (A) and mother (B) near term and estradiol (C) and progesterone (D) in the mother on d 86 and 82 of pregnancy. Fetal data are from all fetuses of six to eight mothers per treatment, and values are expressed as estimated marginal means adjusted for the number of viable pups ± SEM. Maternal data are from six to eight mothers per treatment, and values are expressed as means ± SEM. AA, Amino acids; Chol, cholesterol; d86, d 86 of pregnancy; d62, d 62 of pregnancy; FFA, free fatty acids; Gluc, glucose; Trig, triglycerides. *Asterisks* denote a statistically significant difference compared with the vehicle group, $P < 0.049$.

adiposity at mid-pregnancy (41) and there was a trend to raised maternal circulating glucose concentrations near term. These increased maternal adipose stores were depleted to normal by late pregnancy in the current study, which may have further enhanced nutrient availability for the fetus, either directly or indirectly. This suggests that IGF-II acts on the placenta to increase fetal growth, by sustainedly promoting placental development, but additionally may enhance maternal physiological adaptation to pregnancy.

The mechanism by which increased maternal IGF-I abundance in early to mid-pregnancy sustainedly promotes fetal growth is less clear. The enhanced placental weight at mid-gestation by prior maternal IGF-I treatment (41), which is no longer apparent in late gestation, may have had persistent effects on the fetus that increased fetal growth near term. In addition, unlike IGF-II, IGF-I did not increase maternal fat deposition in mid-pregnancy (41) and in fact reduced fat depot weights near term. Reduced perirenal fat weight was associated with increased maternal circulating progesterone. Reduced adiposity may reflect increased mobilization and/or reduced deposition during pregnancy, which may have increased substrate availability in the maternal circulation for fetal growth. This has been observed in growth hormone-treated pigs where maternal circulating IGF-I concentration was elevated and associated with reductions in weight of maternal backfat depots (67). Another possible explanation is that larger fetuses of IGF-I-treated dams may signal to the mother via nutrient sensors in the fetal circu-

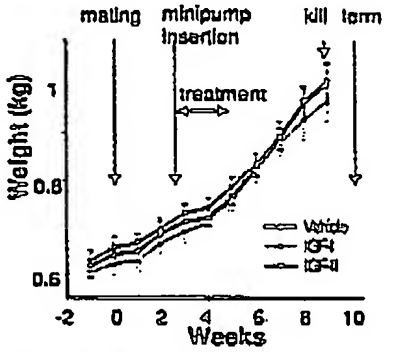

FIG. 7. The effect of exogenous IGFs on maternal weight gain during pregnancy. Female guinea pigs were weighed three times weekly during the study to determine an average weekly weight, from 1 wk before mating and during pregnancy up until kill. Minipumps were inserted on d 20 of pregnancy to deliver vehicle, IGF-I, or IGF-II for 18 d. Term, which is approximately 67–70 d of pregnancy, is denoted on the graph. Data are from seven to nine dams per treatment, and values are expressed as means ± SEM.

lation (such as IGFs and insulin), to influence placental metabolism and increase mobilization of maternal adipose tissue stores late in pregnancy.

These differential IGF effects may reflect their distinct interactions with various receptors, because IGF-I binds with high affinity to the IGF1R but negligibly to IGF2R. In contrast, IGF-II binds to both these receptors, as well as to the insulin receptor. In the current study, during mid-pregnancy, the guinea pig placenta ubiquitously expressed both IGF receptor proteins. More importantly, however, at the time of IGF treatment, IGF1R and IGF2R were localized to the apical surface of trophoblasts, within large maternal blood vessels and blood spaces of the labyrinth. In addition, insulin binding sites have previously been identified in trophoblast of the guinea pig placenta (68–70). This pattern of expression is consistent with the localization of all three receptors to placental trophoblasts in humans and rats (56, 71–77) and abundant expression of IGF1R and IGF2R in invasive trophoblast populations within the human decidua and its vasculature (75).

TABLE 4. Effect of maternal IGF treatment on maternal adipose tissue weights near term

	Vehicle	IGF-I	IGF-II
Number of dams	7	7	9
Weight at d62	978 ± 28	1012 ± 34	971 ± 36
Uterus and contents	242 ± 20	250 ± 44	242 ± 38
Net body mass	736 ± 21	761 ± 52	725 ± 19
Lean body mass	711 ± 18	744 ± 52	702 ± 18
Total fat (g)	25.08 ± 2.3[a]	17.89 ± 1.0[b]	23.14 ± 1.0[a,b]
(% Body weight)	3.4 ± 0.3[a]	2.4 ± 0.2[b]	3.2 ± 0.09[a,b]
Perirenal fat (g)	5.27 ± 0.8	3.50 ± 0.5	4.72 ± 0.4
(% Body weight)	0.71 ± 0.1[a]	0.48 ± 0.08[b]	0.66 ± 0.05[a,b]
Retroperitoneal fat (g)	8.90 ± 0.9[a]	6.27 ± 0.8[b]	8.47 ± 0.6[a,b]
(% Body weight)	1.2 ± 0.1[a]	0.85 ± 0.1[b]	1.2 ± 0.06[a]
Interscapular fat (g)	10.85 ± 0.5[a]	8.11 ± 0.6[b]	9.85 ± 0.4[a,b]
(% Body weight)	1.5 ± 0.1[a]	1.1 ± 0.08[b]	1.4 ± 0.05[a]

Data expressed as means ± SEM. Only tissues that were significantly affected by treatment are shown. Net body mass is weight at postmortem minus the uterus and contents. Lean body mass is not body mass minus total fat. Tissue weight was calculated as a percentage of net body mass. *Different superscripts* denote significant difference between groups, [a] vs. [b], $P < 0.05$.

The specific effects of IGF-II on the placenta, which were not evident in IGF-I-treated animals, suggest that IGF-II actions on the placenta may be mediated by the insulin receptor, which has been implicated in mediating IGF-II effects on fetal growth (78) or by the IGF2R, which it binds with much greater affinity than the IGF1R. There is evidence to suggest that IGF-II acts through IGF2R to promote trophoblast migration and invasion (79), and placental angiogenesis and vascular remodeling (80). IGF-II then, indirectly at least, may enhance placental function by increasing blood supply to the placenta. In contrast, the effects of maternal IGF-I treatment are likely to have been mediated by the IGF1R, particularly because this treatment also reduced IGF-II in the mother.

In conclusion, increased maternal IGF-II in early pregnancy sustainedly promotes placental structural and functional capacity and fetal growth and viability, whereas IGF-I appears to act through the mother to enhance fetal growth to near term. This suggests sustained major and complementary roles in placental and fetal growth for increased circulating IGFs in the mother in early pregnancy.

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Address all correspondence and requests for reprints to: Claire T. Roberts, Research Center for Reproductive Health, Discipline of Obstetrics and Gynecology, University of Adelaide, Adelaide, South Australia, Australia 5005. E-mail: claire.roberts@adelaide.edu.au.

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II. REMARKS

A. Introduction

Applicants submit this Response in a bona fide attempt to (i) advance the prosecution of this case, (ii) answer each and every ground of objection and rejection as set forth by the Examiner, (iii) place the claims in a condition for allowance, and (iv) place the case in better condition for consideration on appeal.

Claims 1-29 are presently pending in the application. As indicated above, Claims 1-5 and 7 has been amended and Claims 6 and 18-29 have been cancelled. Claims 18-17 has previously been withdrawn.

Applicants respectfully submit that the noted amendments merely make explicit that which was (and is) disclosed or implicit in the original disclosure. The amendments thus add nothing that would not be reasonably apparent to a person of ordinary skill in the art to which the invention pertains.

B. Response to Rejections

1. Claim Amendments and Support Therefore

As indicated above, Claim 1, as amended, is based on pending Claim 24 (now cancelled), i.e. the preamble of Claim 24 has been incorporated into Claim 1. The limitation directed to "administration of a differential factor selected from the group consisting of IGF-II, a precursor of IGF-II, an isomer of IGF-II and an analog of IGF-II" has also been deleted and the limitation directed to "administration of an effective amount of IGF-II to a pregnant female mammal in the first half of pregnancy" has been substituted therefore.

Support for Claim 1, as amended, is set forth in the specification, as originally filed, e.g., Example 4 discloses administration of IGF-II to a pregnant female mouse in the first half of pregnancy. Support can also be found in original Claim 5.

Claim 2, as amended, reflects that the "effective amount of IGF-II" comprises an amount sufficient to promote binding of the IGF-II to a cation independent mannose 6 phosphate receptor expressed on a cytotrophoblast cell." Support for Claim 2, as amended, is also set forth in the specification, see, e.g., pp.10-13.

Claims 3-5, as amended, are directed to administration of IGF-II by subcutaneous delivery and/or vaginal pessary. Support for Claims 3-5, as amended, can be found in the specification, as originally filed, and in pending Claim 18. For example, Example 4 provides support for the administration of IGF-II via subcutaneous delivery. The use of vaginal pessaries is disclosed on page 13, line 33 of the specification.

Claim 7, as amended, is directed to the pregnant female mammal being selected from the group consisting of a human, a horse, a cow, a pig, a goat and a sheep. Support for Claim 7 can also be found in the specification, as originally filed, and in pending Claim 7. For example, page 7, lines 12 and 13 of the specification provides suitable mammalian species.

2. 35 U.S.C. §112

The Examiner has rejected Claim 24, which is now embodied in amended Claim 1, under 35 U.S.C. §112, second paragraph, "as being indefinite for failing to particularly point out and distinctly claim the subject matter which the application regards as the invention." The Examiner contends that Claim 24 (now amended Claim 1) does not recite "what the effective amount of the differential factor is supposed to achieve."

As indicated above, Claim 1, as amended, now reflects that administration of an effective amount of the differential factor, i.e. IGF-II, improves a physiological characteristic selected placental growth, placental development and placental differentiation.

3. 35 U.S.C. §102

The Examiner has also rejected Claim 24 (now amended Claim 1) under 35 USC §102(b) as being anticipated by U.S. Pat. No. 5,420,111. The Examiner contends that U.S. Pat. No. 5,420,111 teaches a method of administration of IGF-II to a pregnant female at "any time from conception onward".

It is well established that a rejection for anticipation under § 102 requires that each and every limitation of the claimed invention be disclosed in a single prior art reference. *See In re Paulsen*, 30 F.3d 1475, 1478-79, 31 U.S.P.Q. 2d 1671, 1673 (Fed. Cir. 1994); *Scripps Clinic & Research Foundation v. Genentech, Inc.*, 927 F.2d 1565, 18 U.S.P.Q. 2d 1001 (Fed. Cir.1991). *See also American Permahedge, Inc. v. Barcana, Inc.*, 857 F. Supp. 308, 32 U.S.P.Q. 2d 1801, 1807-08 (S.D. NY 1994) ("Prior art anticipates an invention ... if a single prior art reference contains each and every element of the patent at issue, operating in the same fashion to perform

the identical function as the patent product. ... Thus, any degree of physical difference between the patented product and the prior art, *no matter how slight,* defeats the claim of anticipation."); *Transco Ex parte Levy*, 17 U.S.P.Q. 2d 1461, 1462 (Bd. Pat. App. & Int'l 1990) ("[I]t is incumbent upon the examiner to identify wherein each and every facet of the claimed invention is disclosed in the applied reference".)

Applicants respectfully submit that Claim 1, as amended, and Claims 2-5 and 7, dependent thereon, are not anticipated by U.S. Pat. No. 5,420,111.

U.S. Pat. No. 5,420,111 discloses administration of IGF-I to a pregnant mammal to promote fetal growth. The '111 patent does not disclose that the administration of IGF-II (or IGF-I) improves placental growth, development or differentiation.

In support of the contention that U.S. Pat. No. 5,420,111 discloses administration of IGF-II to a pregnant female mammal, the Examiner relies on the statement in the '111 patent that "although the studies to be discussed herein concentrate on the use of IGF-I, the claims extend to IGF-II and analogues of IGF-I and IGF-II as these are known to exert a similar biological effect to IGF-I (Schoenle et al., Acta Endoc. 108: 167-174, 1985)."

However, it is submitted that one skilled in the art would recognize that the biological effects of IGF-II are ***quite different*** to that of IGF-I (see, e.g., Fowden A. L., "The Insulin-like Growth Factors and Feto-Placental Growth", *Placenta, vol.* 24, pp. 803-812 (2003) and Sferruzi-Perri, et al., "Maternal Insulin-Like Growth Factors-I and –II Act via Different Pathways to Promote Fetal Growth", *Endocrinology,* vol. 147(7), pp. 3344-3355 (2006), copies attached). Thus, one skilled in the art would recognize that while the '111 patent discloses that treatment of IGF-I to a pregnant female mammal may extend to analogues of IGF-I, one skilled in the art would also recognize that the disclosure does not extend to IGF-II.

Applications further submit that U.S. Pat. No. 5,420,111 does not disclose when or how to administer IGF-II to a pregnant female to improve placental growth, placental function, placental development or placental differentiation. Further, the '111 patent does not teach or suggest that the administration of IGF-II to improve placental weight, development or differentiation.

The U.S. Pat. No. 5,420,111 merely discloses that the compositions may be administered "at any time from conception onward" (column 2, last paragraph). Indeed, the '111 patent

discloses that IGF-I is "[d]esirably administered close to the time of birth of the fetus" (column, last paragraph), which teaches away from amended Claim 1.

In addition, given the lack of teaching in U.S. Pat. No. 5,420,111 as to how and when to administer IGF-II, one skilled in the art would recognize that an improvement in placental growth, development or differentiation would not necessarily flow based on the teaching provided in the '111 patent.

U.S. Pat. No. 5,420,111 also teaches away from the current invention by stating that IGF-I has no effect on placental weight (see Example 1). The '111 patent further discloses in Example 3 that since IGF-I does not cross that rat placenta, the effect of IGF-1 is clearly in the maternal compartment (see column 8, 3rd paragraph).

Applicants therefore respectively submit that Claim 1, as amended, is not anticipated by U.S. Pat. No. 5,420,111.

III. CONCLUSION

Applicants, having answered each and every ground of rejection as set forth by the Examiner, and having added no new matter, believe that this response clearly overcomes the references of record and renders the claims clear and definite, and now submit Claims 1-5 and 7 in the above-referenced patent application are in condition for allowance and the same is respectfully solicited.

If the Examiner has any further questions or comments, Applicants invite the Examiner to contact their Attorneys of record at the telephone number below to expedite prosecution of the application.

Respectfully submitted,
FRANCIS LAW GROUP

By: _____
Ralph C. Francis
Reg. No. 38,884

Dated: January 8, 2007
1942 Embarcadero
Oakland, CA 94606
Tel: 510.533.1100

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CURRENT TOPIC

The Insulin-like Growth Factors and feto-placental Growth

Abigail L. Fowden*

Department of Physiology, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK

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The insulin-like growth factors, IGF-I and IGF-II, have an important role in fetoplacental growth throughout gestation. They have metabolic, mitogenic and differentiative actions in a wide range of fetal tissues including the placenta. Both *Igf1* and *Igf2* genes are expressed in fetal tissues. Expression of the *Igf2* gene is more abundant than Igf1 gene expression during mid to late gestation. Both IGF's are also present in the fetal circulations with 3–10 fold higher levels of IGF-II than IGF-I during late gestation. Expression of the *Igf* genes is developmentally regulated in a tissue specific manner and can be affected by nutritional and endocrine conditions *in utero*. Deletion of either *Igf* gene of the *Igf1r* gene retards fetal growth while over-expression of IGF-II leads to fetal overgrowth. In mice, placental growth is affected only by manipulation of the *Igf2* gene. The IGF's also effect the growth of individual fetal tissues and influence the uptake and utilization of nutrients by the fetal and placental tissues. Circulating concentrations and tissue expression of the IGF's are reduced by undernutrition and deficiency of nutritionally sensitive hormones, such as insulin, thyroxine and glucocorticoids. In general, the *Igf1* gene is more responsive to these stimuli than the *Igf2* gene. In addition, the effects of the IGFs on feto-placental growth can be amplified or attenuated by the IGF binding proteins, which are themselves regulated by nutritional and endocrine signals. The *Igf2* gene appears to provide the constitutive drive for intrauterine growth via its placental effects and direct paracrine actions on fetal tissue while the *Igf1* gene regulates fetal growth in relation to the nutrient supply.

Placenta (2003), 24, 803–812

INTRODUCTION

The insulin-like growth factors, IGF-I and IGF-II, have a key role in regulating feto-placental growth throughout gestation. They have metabolic, mitogenic and differentiative actions in a wide range of fetal tissues including the placenta (Jones and Clemmons, 1995). They act as progression factors in the cell cycle and increase DNA synthesis and cell differentiation in cultured embryos and several different fetal cell lines in vitro (Han and Fowden, 1994; Gardner et al., 1999). Their concentrations in the fetus in vivo are positively correlated to birth weight in a number of species including humans, primates, sheep, pigs, rabbits and rodents (Daughaday et al., 1982; Gluckman et al., 1983; Lee, Chung and Simmen, 1993; Tarantal and Gargosky, 1995; Kind et al., 1995; Thakur et al., 2000; Ong et al., 2000). This review examines the relationship between the IGFs and feto-placental growth and places particular emphasis on the expression, action and regulation of the IGFs in fetal and placental tissues. It considers the insulin-like growth factor binding proteins (IGFBPs) in much less detail as their regulation and role in modulating the actions of the IGFs have been reviewed recently (Allan, Flint and Patel, 2001; Schneider et al., 2002; Mohan and Baylink, 2002).

EXPRESSION OF THE IGFS BEFORE BIRTH

In many species, both the *Igf1* and *Igf2* genes are expressed in fetal tissues from the earliest stage of pre-implantation development to the final phase of tissue maturation just before birth (Watson et al., 1991; Hill, Petrik and Arany, 1998; Fowden, Li and Forhead, 1998). During mid to late gestation, *Igf2* gene expression is widespread in fetal tissues and is more abundant than *Igf1* gene expression in rodents, ungulates and humans (Hill, 1990; Delhanty and Han, 1993). Both IGFs are also detected in the fetal circulation from early in gestation but plasma concentrations of IGF-II are 3–10 fold higher than those of IGF-I during late gestation in all species studies so far (Table 1). Tissue and plasma IGF-II are also higher in the fetus than in newborn or adult animals in most species (Gluckman and Butler, 1983; Mesiano et al., 1987). In rodents, IGF-II expression disappears from most tissues except the brain by weaning, with the consequence that IGF-II is virtually undetectable in adult plasma (Lee, Lintar and Efstratiadis, 1990; Singh, Rall and Styne, 1991). In ungulates, *Igf2* gene

* To whom correspondence should be addressed. Tel.: +44-1223-333855; fax: +44-1223-333840; E-mail: alf1000@cam.ac.uk

0143-4004/03/$–see front matter

Table 1. Fetal plasma concentrations of IGF-I and IGF-II during late gestation in different species

	Plasma concentrations (ng/ml)		
	IGF-I	IGF-II	Reference
Human	50–100	150–400	Gluckman et al., 1983
Monkey	70–80	300–400	Tarantal & Gargosky, 1995
Sheep	50–100	400–1000	Owens et al., 1994
Cattle	50–80	280–360	Holland et al., 1997
Pig	20–30	200–300	Lee, Chung and Simmen, 1993
Guinea pig	50–100	500–700	Jones et al., 1987
Rat	50–100	400–700	Daughaday et al., 1982

expression is retained in certain peripheral tissues, such as skeletal muscle after birth and, hence, IGF-II is present in the adult circulation, albeit at lower concentrations than in the fetus (Mesiano et al., 1987; Lee, Chung and Simmen, 1993; Holland et al., 1997). In contrast, tissue expression and plasma level of IGF-I are low in utero compared to postnatal values (Gluckman and Butler, 1983; Mesiano et al., 1987; Singh, Rall and Styne, 1991). Plasma IGF-I levels increase rapidly after birth, primarily as a result of the onset of growth hormone (GH) stimulated IGF-I production by the liver (Gluckman, 1995; Li et al., 1999). There is, therefore, a shift in IGF predominance from IGF-II before birth to IGF-I after birth, which has led to the concept that IGF-II is the IGF primarily responsible for fetal growth (see Gluckman, 1995; Jones and Clemmons, 1995; Allan, Flint and Patel, 2001).

Abundance of the IGF mRNAs varies widely between different fetal tissues and with gestational age. In the sheep fetus, for instance, *Igf2* gene expression is particularly high in the lung and kidney while IGF-I mRNA abundance is highest in liver and skeletal muscle (Delhanty and Han 1993; Kind et al., 1995). Similar differential patterns of IGF expression have also been observed in fetal tissues from rodents and human and non-human primates (Hill, 1990; Lee, Lintar and Efstratiadis, 1990; Lee et al., 2001). The developmental changes in IGF expression are also tissue and IGF specific. In fetal sheep, *Igf1* gene expression is up- and down regulated during late gestation in liver and skeletal muscle, respectively (Fig. 1), while *Igf2* gene expression is suppressed in these tissues and the adrenal, although not in the lung and kidney towards term (Li et al., 1993, 1996; Li et al., 1994; Forhead et al., 2002). The switch from widespread local production of IGF before birth to a more selective pattern of expression after birth, therefore, begins during late gestation before delivery actually occurs. With the transition from parenteral to enteral nutrition at birth, the perinatal switch from local production of predominantly IGF-II to GH dependent production of IGF-I contributes to the resetting of the growth regulatory mechanisms that ensure continued postnatal growth in the new nutritional environment.

In the placenta, expression of the IGFs is species specific. The rodent placenta expresses only the *Igf2* gene while the placenta of guinea pigs, ungulates, human and non-human primates express both *Igf* genes (Lee, Lintar and Efstratiadis, 1990; Lennard, Stewart and Allen, 1995; Han and Carter, 2000). In the latter species, the two IGFs are often localized to specific placental tissues (Lee, Lintar and Efstratiadis, 1990; Han and Carter, 2000). In sheep, IGF-II mRNA is found primarily in fetal mesoderm within the placentomes while IGF-I mRNA is confined to the uterine glands in the intercotyledonary regions (Wathes et al., 1998). In general, IGF-II is expressed in fetal tissue at the fetal-maternal interface of the placenta and in the invading trophoblast in species with invasive placentation (Han and Carter, 2000). Much less is known about the developmental changes in IGF expression in placental than fetal tissues but increased expression of IGF-II has been observed in syncytiotrophoblast and whole villous tissue of primates with increasing gestational age (Zollers et al., 2001). In ruminants, the placenta is both a source of fetal plasma IGF-II and a site for IGF-I clearance from the fetal circulation (Bassett et al., 1990; Holland et al., 1997).

Each of the *Igf* genes has several promoters which leads to multiple mRNA transcripts with different 5' and 3' untranslated regions (Dickson, Saunders and Gilmour, 1991; Gilmour, 1994). These splice variants show developmental and tissue-specific patterns of expression in the fetus (Adamo et al., 1989; Li et al., 1996; Lin and Oberbauer, 1998; Constancia et al., 2000). In sheep, the IGF-I mRNA transcripts are classified as Class 1 or Class 2 depending on whether they are derived from 5' leader exons 1 or 2 (Gilmour, 1994). In adult liver, Class 2 transcripts predominate whereas, in fetal liver, Class 1 is the primary transcript for most of late gestation with little, if any, Class 2 expression until just before term (Figure 1). Similarly, the *Igf2* gene is expressed from at least two promoters in utero in a manner which is tissue specific and dependent on gestational age (Li et al., 1998; Constancia et al., 2000). The *Igf2* gene is also imprinted and expressed only from the paternal allele in the placenta and several fetal tissues excluding the brain (Ferguson-Smith et al., 1991; Miozzo and Simoni, 2002). However, after birth, *Igf2* expression becomes biallelic in tissues, such as the liver, in a number of species including sheep, cattle and humans, although not in mice (DeChiara, Robertson and Efstratiadis, 1990; Kalscheuer et al., 1993; Davies, 1994; McLaren and Montgomery, 1999). Imprinting of *Igf2* is controlled by the *H19* gene, which is itself imprinted and developmentally regulated (Senior et al., 1996; Naimeh et al., 2001). Consequently, there are ontogenic shifts in *Igf2* imprinting and *IGF* gene promotor usage which may influence IGF bioavailability in placental and fetal tissues at critical stages of development.

THE ACTIONS OF THE IGFS ON TISSUE GROWTH AND DEVELOPMENT IN UTERO

In recent years, manipulation of gene expression in mice has been used widely to establish the role of the IGFs in

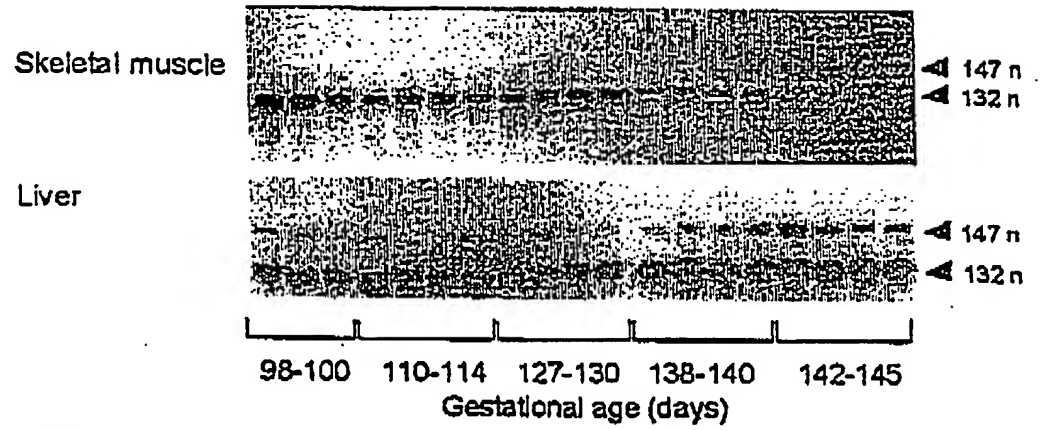

Figure 1. The ontogeny of IGF-1 gene expression in fetal ovine tissues during late gestation. Autoradiograms of RNAase protection assay using ovine IGF-1 ribroprobes with 50 μg total RNA prepared from liver and skeletal muscle of groups of control sheep fetuses aged 100–145 days of gestation (term 145 ± 2 days). Protected probes gave bands at 132 nucleotides (132n) for Class 1 transcripts and at 147 nucleotides (147n) for Class 2 transcripts of the *Igf1* gene. Data from Li et al., 1995, 2002.

Table 2. The effects of disruption of genes controlling IGF bioavailability on fetal and placental weights in mice during late gestation (>85%)

Gene target	Effect	Per cent of normal weight		Reference
		fetus (%)	Placenta (%)	
Igf1	No tissue or plasma IGF-I	60	100	Baker et al., 1993
Igf2	No tissue or plasma IGF-II	60	75	DeChiara, Robertson and Efstratiadis, 1990
Placental P0 *Igf2*	Decrease placental IGF-II, Normal fetal IGF-II	75	65	Constancia et al., 2002
IGF-type 1 receptor (*Igf1r*)	No action of IGF-I/IGF-II at IGF1r	45	100	Baker et al., 1993
GF-type 2 receptor (*Igf2r*)	No IGF-II clearance, Increased plasma IGF-II	140	140	Ludwig et al., 1996
H19	No suppression of maternal *Igf2* allele, Increased tissue IGF-II	130	140	Lau et al., 1994
Igf2r and *H19*	Increased tissue and plasma IGF-II	200	230	Eggenschwiler et al., 1997

feto-placental growth (Efstratiadis, 1998). Deletion of either the *Igf1* or *Igf2* gene retards fetal growth to a similar extent (Table 2). When both genes are deleted simultaneously, the effects on fetal growth are additive and the double mutants are only 30 per cent of the normal bodyweight at term (Efstratiadis, 1998). Deletion of the IGF-type I receptor gene (*Igf1r*) produces a more severe growth retardation than seen in either the *Igf1* or *Igf2* nulls (Table 2) which suggests that both IGFs act through the type 1 IGF receptor to stimulate tissue accretion (Efstratiadis, 1998). Conversely, fetal growth is enhanced by IGF-II over-expression caused either by deletion of the IGF-type 2 clearance receptor (*Igf2r* null) or by biallelic IGF-II expression in response to *Igf2* imprint relaxation induced by disruption of the *H19* gene (Table 2; Lau et al., 1994; Ludwig et al., 1996). fetal overgrowth is greatest in the double *Igf2r/H19* mutants, which have the highest IGF-II levels and the largest placentae (Table 2; Eggenschwiler et al., 1997). In the human, homozygous partial deletion of the IGF-1 gene is also associated with failure of growth, both in utero and postnatally (Woods et al., 1996).

These IGF-induced changes in fetal bodyweight are accompanied by abnormalities in the development of individual fetal tissues (Woods et al., 1996; Efstratiadis, 1998). The *Igf1* and *Igf2* null mice were both viable although they showed delayed ossification and general dwarfism at birth. The growth rate of the *Igf1*, but not *Igf2* null mice remained low after birth which is consistent with the loss of IGF-II expression in wild types after weaning (see Miozzo and Simoni, 2002). Deletion of the IGF type I receptor had more widespread effects on murine tissue growth and lead to delayed ossification, thin skin and hypoplasia of respiratory and other muscles, which proved fatal at birth (Efstratiadis, 1998). Over-expression of IGF-II caused generalized organomegaly with kinky tails, extra toes, oedema and cardiac abnormalities

and was usually lethal at birth (Lau et al., 1994; Louvi, Accili and Efstratiadis, 1997). Similarly, in sheep produced in vitro or by cloning, increased IGF-II exposure induced by reduced *Igf2r* gene expression is associated with multiple developmental abnormalities, muscle hypertrophy and generalized overgrowth of the fetus (Young et al., 2001).

In mice, placental growth is affected by manipulation of the *Igf2*, but not the *Igf1* or *Igf1r* genes (Table 2). The placenta is growth retarded by 30–40 per cent in mice that lack IGF-II either in all placental cell types (*Igf2* null, DeChiara, Robertson and Efstratiadis, 1990) or in the labyrinthine trophoblast cells specifically (P0 null, Constancia et al., 2002). In P0 mutants, the placenta is small but morphologically normal whereas, in *Igf2* nulls, placental growth retardation is accompanied by structural abnormalities, particularly in the glycogen cells (Rossant and Cross, 2001; Constancia et al., 2002). Conversely, placentomegaly occurs when IGF-II is over-expressed by changes in IGF-II clearance or *Igf2* imprinting (Table 2). The growth stimulatory effects of IGF-II on the placenta may be paracrine and/or endocrine but do not appear to be mediated via the IGF type 1 receptor (Table 2). Placental growth is also normal in double mutants lacking both IGF type 1 and insulin receptors which suggests the IGF-II may act through an unknown placental specific receptor (Louvi, Accili and Efstratiadis, 1997). The existence of another type of IGF receptor in the placenta may also explain the unusual characteristics of IGF-1 binding observed in the ovine trophoblast between 45–75 days of gestation when no *Igf1r* gene expression can be detected in the placentomes (Lacroix, Servely and Kann, 1995; LeRoith et al., 1995; Wathes et al., 1998). However, whether this placental specific IGF receptor is responsible for placentomegaly in mice during IGF-II over-exposure remains unknown.

In *Igf2* nulls, placental and fetal growth retardation occurs in parallel and begins around mid gestation (Baker et al., 1993). In P0 mutants lacking IGF-II only in the labyrinthine placenta, growth retardation of the placenta begins at a similar stage but growth of the fetus is not slowed until much later in gestation (Constancia et al., 2002). At term, the weight of the fetus produced per gram of placenta was greater in P0 mutants than in wild types although both the P0 placenta and fetus were smaller than normal at this stage. These observations suggest that IGF-II may affect the functional capacity of the placenta to transfer nutrients as well as placental size. Both IGFs have been shown to alter glucose and amino acid transfer across cultured human trophoblast derived from chorionic villi (Kniss et al., 1994). Similarly, administration of IGF-1 to either the fetus or mother has been shown to alter the transfer and partitioning of glucose and amino acids between ovine fetal and uteroplacental tissues (Harding et al., 1994; Liu et al., 1994). Changes in expression of the amino acid transporter proteins have been observed in specific regions of the *Igf2* null placenta (Matthews et al., 1999). Measurement of passive and secondarily active transport across the P0 mutant placenta has shown that passive diffusion is reduced while System A amino acid transport is increased per unit surface area of placenta throughout late gestation (Constancia et al., 2002). Up-regulation of System A amino acid transport, therefore, appears to compensate for the smaller size of the P0 placenta for much of gestation and only fails to meet the growth requirements of the fetus late in gestation (Reik et al., 2003). Whether this up-regulation of amino acid transport is the consequence of a paracrine IGF-II deficiency in the labyrinthine placenta or of an endocrine action of the normal circulating levels of the IGF-II in the P0 fetus has yet to be determined.

While gene manipulation experiments have shown that IGF-I affects fetal growth directly, they suggest that the growth-promoting actions of IGF-II on the fetus may be indirect and mediated via changes in the growth and nutrient transport capacity of the placenta (Table 2). However, more detailed comparison of the growth rates of various IGF mutants has shown that fetal growth is determined by the actions of IGF-I on the IGF type 1 receptor and of IGF-II on both the IGF type 1 and insulin receptors (Eggenschwiler et al., 1997; Louvi, Accili and Efstratiadis, 1997; Efstratiadis, 1998). The growth-promoting action of IGF-II was predominantly through the IGF type 1 receptor, although insulin receptor mediated action increased during late gestation to account for about 40 per cent of the total IGF-II activity at term (Louvi, Accili and Efstratiadis, 1997). The interactions of IGF-I and IGF-II with the IGF type 1 receptor were equally as important in determining fetal growth during late gestation (Baker et al., 1993).

Administration of IGF-I directly to sheep and monkey fetuses for 10 days has no effect on placental or fetal body weight (Lok et al., 1996; Tarantal, Hunter and Gargosky, 1997). However, in both species, IGF-1 increased the weight of specific fetal organs such as the spleen, thymus and kidney. It also increased the weight of the liver, lungs heart, pituitary and adrenal glands in the sheep fetus (Lok et al., 1996). In addition, IGF-1 administration promoted skeletal maturation in the sheep fetus during late gestation (Lok et al., 1996). More long-term administration of IGF-I via the gut (30 days) has been shown to increase total bodyweight in growth retarded sheep fetuses (Kimble et al., 1999). These changes in growth of the internal organs and skeleton are probably the result of the anabolic actions of IGF-I on fetal metabolism. Short-term infusion of IGF-I (4 h) into the sheep fetus has been shown to increase placental amino acid transfer and to decrease proteolysis and amino acid oxidation in fetal tissues (Harding et al., 1994; Boyle et al., 1998; Jensen et al., 2000). This would increase the availability of amino acids for protein synthesis and the accretion rate of protein in the fetal carcass. However, IGF-I administration reduces the fetal plasma concentration of insulin (Leichty et al., 1996), a major promoter of fetal growth (Fowden, 1995). It also suppresses *Igf1* and *Igf2* gene expression in fetal ovine liver which may reduce the paracrine stimulus for tissue growth (Kind et al., 1996). Changes in insulin secretion and local IGF production may therefore explain the selective effects of IGF-I administration on tissue growth in sheep and monkey fetuses.

Table 3. The effects of manipulating the fetal nutrient supply on fetal IGF concentrations

Treatment	Species	Per cent change in plasma IGF: IGF-I (%)	IGF-II (%)	Reference
Maternal nutrition				
Protein deprivation	Rat	↓50–60	No change	Muzka et al., 1995
Fasting	Rat	↓60–70	↓10	Straus et al., 1991
	Sheep	↓50	↓15–20	Oliver et al., 1996; Lee et al., 1997
Restrict uterine blood flow	Rat	↓50	No change to ↑10	Price et al., 1992
	Guinea pig	↓70	No change	Jones et al., 1987
	Sheep	↓50	↓20	McLellan et al., 1992
Restrict placental function				
Carunclectomy	Sheep	↓70–75	No change to ↓20	Owens et al., 1994
Cord occlusion—partial	Sheep	No change	No change	Green et al., 2000
—complete	Sheep	↓80	No change	Bennet et al., 2001
Maternal hypoxia	Rat	↓10	↑40	Tapanainen et al., 1994
	Sheep	↓40–50	No change	Iwamoto et al., 1992

As well as stimulating cell proliferation, IGF-I and IGF-II have been shown to prevent apoptosis in cultured cell lines (Han and Fowden, 1994; Allan, Flint and Patel, 2001). In rodents, the β cells of the endocrine pancreas undergo programmed apoptosis followed by a wave of islet neogenesis around the time of weaning (see Hill, Petrik and Arany, 1998). This sequence of β cell destruction and renewal coincides with a decrease in pancreatic *Igf2* gene expression and with a switch from fetal β cells capable of replication to non-proliferating β cells with insulin secretory responses characteristic of the adult (see Fowden and Hill, 2001). When IGF-II levels are maintained during weaning by transgenic over-expression of IGF-II, the wave of apoptosis does not occur and β cell mass increases five fold (Hill, Petrik and Arany, 1998). These observations suggest that IGF-II may have a key role in cell differentiation, particularly during the perinatal period when many tissues are adapting to new environmental conditions. Certainly, in the sheep fetus, the decline in *Igf2* gene expression in the liver, muscle and adrenal towards term coincides with the main phase of prepartum structural and functional maturation in these tissues (Li et al., 1993, 1996, 2002; Lü et al., 1994).

REGULATION OF IGF EXPRESSION

Nutritional regulation

fetal IGF concentrations are affected by a wide range of experimental manipulations which alter the placental supply of nutrients to the fetus (Table 3). Reduced availability of both substrates and oxygen or of either substrate or oxygen alone lower fetal IGF concentrations (Table 3). Nutrient restriction has a more pronounced effect on circulating levels of IGF-I than IGF-II, irrespective of the cause or nature of the nutrient deficit (Table 3). Similarly, there is a greater reduction in tissue abundance of IGF-I than IGF-II mRNA during nutrient restriction in fetal rats and sheep (Strauss et al., 1991; Kind et al., 1996; Bramall et al., 2000). In fetal sheep, IGF-I, but not IGF-II concentrations are directly correlated with the fetal arterial blood pO_2 and glucose levels during late gestation (Carr et al., 1995). Indeed, IGF-I levels can be raised in the fetus of fasted ewes by direct fetal infusion of either glucose or insulin (Oliver et al., 1996). Since insulin increases glucose uptake by fetal tissues (Fowden, 1995), these observations suggest that IGF-I is regulated by the cellular availability of glucose (Fowden, Li and Forhead, 1998). In contrast, fetal levels of IGF-II are reduced only during the severest types of growth retardation or when nutrient deprivation is particularly extreme or prolonged (Owens et al., 1994; Holmes et al., 1997). The *Igf1* gene, therefore, appears to be more responsive to changes in nutritional state than the *Igf2* gene in the fetus during late gestation. These observations are consistent with the findings that birth weight is more closely correlated with plasma IGF-I than IGF-II in several species (Carr et al., 1995; Ong et al., 2000).

Endocrine regulation

fetal IGF concentrations are also affected by the endocrine environment in utero, particularly by nutritionally sensitive hormones known to regulate fetal development, such as insulin, thyroxine and glucocorticoids (Fowden, 1995). Like nutrient restriction, deficiency of these hormones in utero affects expression of IGF-I more readily than IGF-II. Compared to the adult, GH has relatively little effect on the IGF axis in the fetus, probably due to the paucity of GH receptors in fetal tissues for most of gestation (Gluckman, 1995; Fowden, Li and Forhead, 1998). Insulin deficiency, on the other hand, reduces plasma IGF-I, but not IGF-II levels in the sheep fetus (Gluckman et al., 1987). Conversely, insulin infusion raises plasma IGF-I, but has no effect on IGF-II levels (Oliver et al.,

1996). fetal insulin and IGF-I levels are, therefore, positively correlated over the normal range of concentrations observed in utero and act synergistically to enhance accumulation of glucose and amino acid carbon, respectively, in the fetal tissues (Owens, 1991; Fowden, 1995; Han and Fowden, 1994).

In fetal sheep and pigs, circulating IGF-I, but not IGF-II concentrations are also reduced by thyroid hormone deficiency and are restored to normal values by thyroxine treatment (Mesiano et al., 1989; Latimer et al., 1993). The low levels of IGF-I induced by hypothyroidism were accompanied by fetal growth retardation (Fowden, 1995) and by tissue-specific changes in *Igf1*, but not *Igf2* gene expression (Latimer et al., 1993; Forhead et al., 1998, 2000). In fetal pigs, thyroid hormone deficiency reduced the IGF-I content of a wide range of fetal tissues, including the liver and skeletal muscle (Latimer et al., 1993). In contrast, thyroidectomy of the sheep fetus increased IGF-I mRNA levels in the liver, but reduced its abundance in skeletal muscle during late gestation (Forhead et al., 2000, 2002). Hypothyroidism also altered the normal ontogenic pattern of *Igf1* gene expression in both these tissues towards term (Forhead et al., 2000, 2002). Hence, thyroid hormone mediated changes in *Igf1* gene expression probably have an important role in regulating fetal growth, particularly in tissues, such as skeletal muscle, which normally accounts for 25–33 per cent of fetal bodyweight at term (Owens, 1991). However, the effects of thyroid hormones on placental development and *Igf* gene expression remain largely unknown.

In contrast to insulin and the thyroid hormones, glucocorticoids affect expression of both *Igf* genes, although their effects are tissue and IGF specific (Fowden, Li and Forhead, 1998). In fetal sheep, cortisol up- and down-regulates *Igf1* gene expression in liver and skeletal muscle, respectively, whereas, it down-regulates *Igf2* gene expression in these tissues (Figure 2). These changes in tissue expression occur both in response to exogenous cortisol infusion before term and when fetal cortisol levels rise endogenously during the immediate prepartum period (Figure 2). The cortisol induced changes in tissue *Igf* gene expression are also accompanied by decreases in the fetal growth rate and, close to term, by a fall in plasma IGF-II levels (Gluckman et al., 1983; Fowden et al., 1996). Cortisol, therefore, appears to initiate the switch from paracrine IGF production in utero to the hepatic production of endocrine IGF-I characteristic of the postnatal animal. However, the mechanisms by which cortisol acts remain unclear. Cortisol has been shown to suppress transcription of the ovine *Igf2* gene via specific promotors in fetal liver in vivo and in cell lines in vitro (Li et al., 1998). In contrast, the ovine *Igf1* gene contains no recognizable glucocorticoid response elements (Dickson, Saunders and Gilmour, 1991). Hence, cortisol may act on *Igf* gene expression either directly or indirectly through changes in GH receptor gene expression (Li et al., 1999) and/or via other transcription factors or cortisol-dependent hormones, such as triiodothyronine (Forhead et al., 1998, 2002). Whether the prepartum cortisol surge is also involved in the perinatal transition from monoallelic to biallelic *Igf2* gene expression remains unknown.

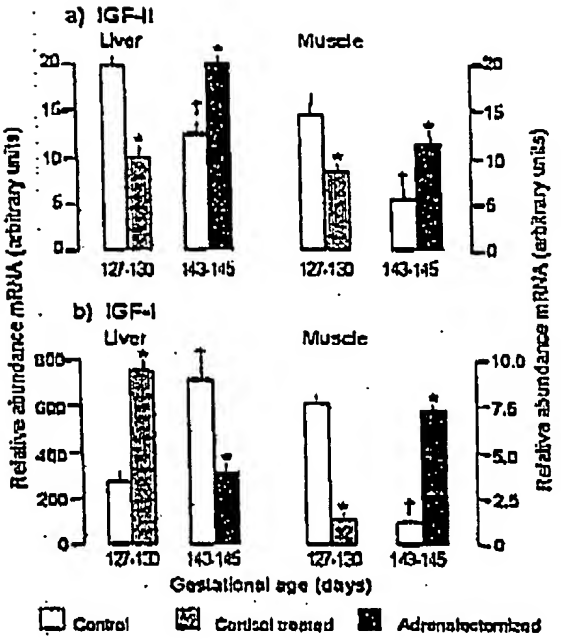

Figure 2. The control of IGF gene expression in fetal ovine tissues by cortisol during late gestation. Cortisol levels were manipulated before term by cortisol infusion and at term by fetal adrenalectomy. The figure shows mean (±SE) abundances of (a) IGF-II mRNA and (b) IGF-I mRNA in liver and skeletal muscle from sheep fetus delivered either before term (127–130 days) after 5 days of infusion of saline (open columns, controls n=5 low cortisol values) or cortisol (grey columns, 2 mg/kg/day, n=5, high cortisol values) or at (143–145 days) with (open columns, controls n=4, high cortisol values) or without adrenal glands (black columns, adrenalectomized n=4, low cortisol values). *Significantly different from value in the age-matched control group $P<0.05$, †significantly different from value in control fetuses at 127–130 days, $P<0.05$. Data from Li et al., 1993, 1996, 2002.

Increases in fetal plasma cortisol also occur before term during adverse intrauterine conditions, such as hypoxaemia and undernutrition (Challis et al., 2001). Although these increments tend to be smaller than those seen at term, they may explain, in part, the changes in tissue *Igf1* gene expression observed during nutrient restriction (Table 3). The ability of glucocorticoids to suppress *Igf2* gene expression in certain fetal tissues is also consistent with the observations that fetal IGF-II levels only fall close to term and during the severest types of growth retardation when fetal cortisol levels are high. Indeed, glucocorticoid-dependent changes in *Igf2* gene expression may be the major mechanism regulating IGF-II availability in the fetus during late gestation.

IGFBP regulation

The bioavailability of the IGFs is also affected by the tissue expression and circulating concentrations of the IGFBPs

(Jones and Clemmons, 1995) and of the soluble form of the IGF-II receptor, which binds up 40 per cent of the IGF-II in fetal ovine plasma (Gallaher et al., 1994). At least six different IGFBPs have been identified in fetal plasma and tissues, each of which has a unique pattern of expression (Jones and Clemmons, 1995; Allan, Flint and Patel, 2001). In rodents, ungulates, humans and non-human primates, the most prevalent IGFBPs in fetal plasma and tissue are the IGFBPs 1 to 4, although their relative abundance varies both within and between species (Donovan et al., 1989; Lee, Chung and Simmen, 1993; Carr et al., 1995; Kind et al., 1995; Tarantal and Gargosky, 1995; Osorio et al., 1996). fetal expression of these IGFBPs is also tissue specific and developmentally regulated in most species studied (Donovan et al., 1989; Delhanty and Han, 1993; Lee, Chung and Simmen, 1993; Carr et al., 1995; Tarantal and Gargosky, 1995).

In sheep and humans, fetal bodyweight at term is positively correlated to plasma IGFBP-3, but inversely related to plasma IGFBP-1 over the normal range of birthweights (Carr et al., 1995; Kind et al., 1994; Kajantie et al., 2001). When intrauterine growth is retarded in human infants, plasma concentrations of IGFBP-1 and -2 are elevated while IGFBP-3 levels are reduced compared to the values found in normally grown infants of the same gestational age (Lassarre et al., 1991; Chard, 1994; Ong et al., 2000). Similarly, hepatic expression and plasma levels of IGFBP-1 are increased in growth retarded rat pups during late gestation (Strauss et al., 1991; Price et al., 1992). Transgenic over-expression of IGFBP-1 and -3 in mice also retards growth, both pre- and post-natally (Silha and Murphy, 2002). Changes in IGFBP expression, therefore, have an important role in modulating the growth-promoting actions of the IGFs, although identifying the specific effects of each IGFBP is difficult because of their functional redundancy (Allan, Flint and Patel, 2001; Silha and Murphy, 2002).

During late gestation, IGFBP expression in the fetus is affected by both the nutritional and endocrine conditions in utero. Generally, these conditions have more pronounced effects on IGFBP-1, -2 and -4 than IGFBP-3. Tissue expression and plasma levels of IGFBP-1 are elevated in rat and sheep fetuses by fetal nutrient restriction induced by maternal dietary restriction, reduced uterine blood flow or by occlusion of the umbilical cord (Strauss et al., 1991; Price et al., 1992; Osborn et al., 1992; Hooper et al., 1994; Bennet et al., 2001). Conversely, increasing fetal glucose levels lowers hepatic expression and plasma IGFBP-1 in fetal sheep (Osborn et al., 1992). In contrast, levels of the soluble form of the IGF-II type 2 receptor are lowered by fetal undernutrition and raised by fetal hyperglycaemia (Gallaher et al., 1994). Specific fetal hypoxaemia has also been shown to increase IGFBP-1 levels in fetal ovine plasma (Iwamoto et al., 1992). Similarly, in human infants, IGFBP-1 levels are higher in hypoxic than normoxic neonates at birth (Chard, 1994). The increase in fetal IGFBP-1 expression observed during adverse conditions may attenuate the growth-promoting effects of the IGFs and, thereby, contribute to the decline in fetal growth rate found in these circumstances. In contrast, the fall in the soluble form of the IGF-II type 2 receptor during fetal undernutrition may increase availability of plasma IGF-II and promote tissue differentiation, while maintaining a basal stimulus to fetal growth in the face of low IGF-1 bioavailability.

The nutritionally induced alterations in fetal IGFBP expression may be due, in part, to the concomittant changes in the fetal endocrine environment. In fetal ungulates, hepatic expression and plasma concentrations of IGFBP-1 are reduced by insulin and increased by catecholamines and thyroxine (Latimer et al., 1993; Gallaher et al., 1994; Hooper et al., 1994). Furthermore, since the ontogenic changes in IGFBP expression closely parallel the normal prepartum rise in plasma cortisol in the sheep fetus (Carr et al., 1995; Fowden, Li and Forhead, 1998), glucocorticoids may also be involved in regulating IGFBP production in utero as occurs in postnatal animals (Allan, Flint and Patel, 2001). Certainly, in human infants, antenatal glucocorticoid treatment lowers plasma IGFBP-1 and raises plasma IGFBP-3 concentrations at delivery (Kajantie et al., 2001).

The effects of the glucocorticoids on the IGF axis may provide a mechanism for the intrauterine programming of adult disease. Human epidemiological observation and experimental studies on animals have shown that impaired intrauterine development is associated with postnatal abnormalities in cardiovascular and metabolic function, which, in humans, lead to an increased incidence of adult-onset degenerative diseases, such as coronary heart disease and Type II diabetes (Barker, 2001; Bertram and Hanson, 2001). Precocious elevations in fetal plasma cortisol induced by sub-optimal conditions in utero may cause a premature transition from IGF-II to IGF-I production with beneficial effects on tissue differentiation should delivery occur before full term. However, if delivery is not stimulated prematurely, the cortisol-induced switch from the fetal to the adult mode of somatotrophic regulation may lead to inappropriate changes in cell proliferation and differentiation in utero with adverse sequelae both at birth and much later in life.

CONCLUSIONS

Both *Igf* genes have important roles in feto-placental growth but their expression and specific actions differ. Their effects can also be amplified or attenuated by the IGFBPs. Although *Igf1* gene expression is low in the fetus, IGF-1 appears to have a more prominent role than IGF-II in modulating cell proliferation in relation to the specific endocrine and nutritional conditions prevailing in utero (Figure 3). Tissue expression and circulating levels of IGF-1 are regulated by the nutrient supply and enhance the uptake and utilization of substrates by the fetal tissues. This anabolic effect of IGF-I, particularly on fetal amino acid metabolism leads to tissue accretion and growth of the fetus (Figure 3). fetal IGF-1, therefore, stimulates fetal growth when nutrients are available and, thereby, ensures that the fetal growth rate is commensurate with the

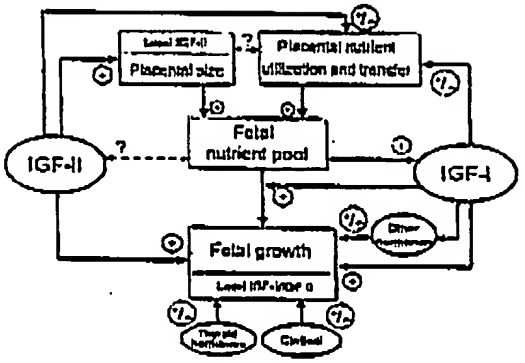

Figure 3. Schematic diagram showing the role of fetal IGF-I and IGF-II in the control of feto-placental growth. Solid line=known effects, dotted line=possible effects, + positive effects, − inhibitory effects, O circulating hormones, □ physiological systems.

nutrient supply. In part, the actions of IGF-I on tissue growth may be mediated by changes in other growth regulatory fetal hormones (Figure 3). fetal IGF-I has also been shown to modify placental nutrient utilization and transfer but appears to have relatively little effect on placental size (Figure 3). The *Igf2* gene, on the other hand, is highly expressed in-utero and has a major role in placental growth and nutrient transfer (Figure 3). It appears to provide the constitutive drive for intrauterine growth via both its placental effects and direct paracrine actions in the fetal tissues. Compared to *Igf1*, the *Igf2* gene is relatively unresponsive to nutritional stimuli. However, in specific fetal tissues, it does respond to changes in the fetal glucocorticoid concentration (Figure 3). fetal IGF-II may, therefore, be responsible for the developmental and tissue specific changes in cell differentiation that occur in key tissues close to term and during adverse intrauterine conditions when fetal cortisol levels are high.

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Maternal Insulin-Like Growth Factors-I and -II Act via Different Pathways to Promote Fetal Growth

Amanda N. Sferruzzi-Perri, Julie A. Owens, Kirsty G. Pringle, Jeffrey S. Robinson, and Claire T. Roberts

Research Center for Reproductive Health, Discipline of Obstetrics and Gynecology, University of Adelaide, Adelaide, South Australia 5005, Australia

The placenta transports substrates and wastes between the maternal and fetal circulations. In mice, placental IGF-II is essential for normal placental development and function but, in other mammalian species, maternal circulating IGF-II is substantial and may contribute. Maternal circulating IGFs increase in early pregnancy, and early treatment of guinea pigs with either IGF-I or IGF-II increases placental and fetal weights by mid-gestation. We now show that these effects persist to enhance placental development and fetal growth and survival near term. Pregnant guinea pigs were infused with IGF-I, IGF-II (both 1 mg/kg·d), or vehicle sc from d 30–38 of pregnancy and killed on d 62 (term = 69 d). IGF-II, but not IGF-I, increased the mid-sagittal area and volume of placenta devoted to exchange by approximately 30%, the total volume of trophoblast and maternal blood spaces within the placental exchange region (+29% and +46%, respectively), and the total surface area of placenta for exchange by 89%. Both IGFs reduced resorptions, and IGF-II increased the number of viable fetuses by 26%. Both IGFs increased fetal weight by 11–17% and fetal circulating amino acid concentrations. IGF-I, but not IGF-II, reduced maternal adipose depot weights by approximately 30%. In conclusion, increased maternal IGF-II abundance in early pregnancy promotes fetal growth and viability near term by increasing placental structural and functional capacity, whereas IGF-I appears to divert nutrients from the mother to the conceptus. This suggests major and complementary roles in placental and fetal growth for increased circulating IGFs in early to mid-pregnancy. (*Endocrinology* 147: 3344–3355, 2006)

THE PLACENTA IS a multifunctional organ that forms the interface between the fetal and maternal circulations. It is essential for fetal growth as it supplies the developing fetus with oxygen and nutrients, transporting them from the mother into the umbilical circulation. Abnormalities in placental structural development can impair placental function, reducing substrate supply to the fetus, and may result in intrauterine growth restriction (1). It is estimated that placental dysfunction accounts for 70–80% of growth-restricted newborns (2), currently affecting 6% of pregnancies in developed countries (3) and up to 40% in developing countries (4). Intrauterine growth restriction is associated with perinatal morbidity and mortality (5, 6) and increases the risk of poor health in childhood and adult life (7). In addition, impaired placental trophoblast invasion of the maternal uterine vasculature and/or poor placental function are implicated in other major pregnancy complications, such as miscarriage (8), preeclampsia (1), placental abruption (9), and preterm labor (10, 11). Therefore, it is imperative that we understand the factors essential for regulating placental functional development to identify causes of such diseases and as a basis for the development of therapeutics.

The IGF-I and -II have been implicated in placental structural and functional development. *Igf2* overexpression in mice causes placental and fetal overgrowth (12), whereas *Igf2* gene deletion reduces placental weight by 17% on d 13.5 and 25% on d 16.5 of gestation, with a fetal weight reduction of 40% from d 16.5 (term = 19 d) (13, 14). In addition, placental amino acid transporter expression is altered by *Igf2* deficiency in mice (15). Ablation of the placental-specific *Igf2* promoter (P0) in mice reduces placental weight and adversely affects placental structural differentiation and transport capacity, with reduced fetal weight evident 2 d later (16, 17). The latter reduction in fetal weight was comparable to that induced by global *Igf2* gene ablation, suggesting that the effects of *Igf2* deficiency on fetal growth are mediated by actions on the placenta in mice.

In contrast, *Igf1* gene ablation in mice does not alter placental weight but reduces fetal weight, indicating that IGF-I is important in the fetus (14, 18). IGF-I may modulate placental nutrient capacity because IGF-I administration to pregnant rats, or increased endogenous expression in pregnant mice, increases the weight of the fetus but not that of the placenta (19). IGF-I stimulates glucose and amino acid uptake in cultured human placental trophoblasts (20–22) and promotes placental nutrient uptake and metabolism when infused into fetal sheep (23–25). Moreover, exposure to IGF-I inhibits release of vasoconstrictors, such as thromboxane B2 and prostaglandin F2 α, in human term placental explants (26), which may increase placental blood flow and delivery of nutrients for the growth of the fetus.

The placenta is exposed to IGFs from multiple sources, including those produced locally and those circulating within the fetus and mother. Maternally derived IGFs may have a major influence on placental development, particularly in women and in guinea pigs where circulating IGFs are substantial (27, 28). Indeed, the IGF axis in guinea pigs is very similar to that of humans (29), whereas rats and mice do not

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Abbreviation: IGFBP, IGF binding protein.

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have circulating IGF-II postnatally. The placenta in guinea pigs is more similar to the human placenta than that of other nonprimate species being hemomonochorial, although it is labyrinthine rather than villous in structure. The guinea pig placenta is comprised of a labyrinth, which contains both fetal capillaries and maternal blood sinuses and provides the means for exchange between the two circulations and an interlobium that is comprised of syncytiotrophoblast and maternal blood sinuses, and is the site where much of the metabolic activity of the placenta is thought to occur (30). In the human placenta, exchange and endocrine functions are performed in the placental villi (31). In addition, placental trophoblast cells in guinea pigs are highly invasive and, like those in humans, engage in interstitial and endovascular invasion of the decidua. They remodel the uterine spiral arterioles to permit the large increase in blood flow to the placenta (32, 33) that is essential for placental growth and subsequent function and therefore fetal growth.

In the guinea pig, major structural determinants of placental function are strongly predicted by maternal IGF-II concentration in mid-pregnancy and by maternal IGF-I in late pregnancy (34, 35). Furthermore, in this species, food restriction reduces maternal plasma IGF concentrations (36) that correlate with delayed structural and functional maturation of the placenta and with reduced fetal growth (34, 35, 37). The structural defects in the placenta of food-restricted guinea pigs are similar to those seen in placentas from women with preeclampsia (34). In addition, reduced maternal plasma IGF-I in pregnant women is associated with placental dysfunction and small-for-gestational-age (38, 39) or growth-restricted infants (40).

Consistent with these adaptive changes in maternal IGFs regulating placental development, maternal supplementation with IGF-I or IGF-II in early to mid-pregnancy in the guinea pig increases placental and fetal weights by mid gestation (41). Therefore, we suggest that the increased maternal production of both IGFs in early pregnancy is an important adaptation to pregnancy, which promotes placental functional development and consequently fetal growth. Whether anabolic effects of an increased abundance of maternal IGFs in early pregnancy on the placenta would persist into late gestation and affect the fetus is currently unknown. Therefore, the aim of this study was to determine the effects of maternal IGF-I and -II supplementation in early to mid-pregnancy on placental development and fetal growth and viability near term.

Materials and Methods

Animals

This study was approved by the University of Adelaide, Animal Ethics Committee. Virgin guinea pigs (IMVS colored strain, approximately 500 g, 3–4 months old) were housed individually in the University of Adelaide Medical School Animal House. Guinea pigs were provided with food and water *ad libitum*. Females were examined daily for estrus indicated by a ruptured vaginal membrane (complete estrous cycle lasts approximately 15 d) and mated naturally with a male. The day a copulatory plug was observed was designated as d 1 of pregnancy. From 2 wk before mating, body weight was monitored three times weekly. Females were assigned to three groups of similar mean weight at mating.

On d 20 of pregnancy (term 69–70 d), females were anesthetized with atropine sulfate (0.05 mg/kg, sc; Apex Laboratories, Sydney, Australia), xylazine hydrochloride (4 mg/kg, im; Troy Laboratories, Sydney, Australia), ketamine hydrochloride (25 mg/kg, ip; Troy Laboratories) and administered local analgesia with lignocaine hydrochloride (Troy Laboratories). A 200-μl mini osmotic pump (Alzet 2002; Alzet, San Francisco, CA) was surgically inserted sc. Minipumps had previously been prepared to deliver vehicle (0.1 M acetic acid) ($n = 7$) or 1 mg/kg·d IGF-II ($n = 7$) or IGF-I ($n = 9$) (human recombinant protein; GroPep Pty. Ltd., Adelaide, Australia) for 18 d at a flow rate of 0.51 μl/h.

On d 62 of pregnancy, guinea pigs were killed by overdose of sodium pentobarbitone (Lethabarb; Virbac, Sydney, Australia). Viable and resorbing implantation sites were counted and the uterus and its contents, viable fetuses, and placentae were weighed. Fetal biparietal diameter, abdominal circumference, and crown-to-rump length were measured. A 3-mm mid-sagittal placental slice was fixed in 4% paraformaldehyde for structural analysis. Analyses of body composition were performed on the mothers and all fetuses to determine the absolute and relative weights of adrenals, kidneys, pancreas, liver, spleen, heart, brain, lungs, gastrointestinal tract, reproductive tract, biceps, triceps, gastrocnemius and soleus muscles and retroperitoneal, perirenal, and interscapular adipose tissues. Skin and carcass weights of the dams and carcass weight of the fetuses were also recorded.

Measurement of maternal circulating IGF-I, IGF-II, and IGF binding proteins (IGFBPs)

In an additional cohort of guinea pigs (vehicle, $n = 5$; IGF-I, $n = 5$; IGF-II, $n = 3$), mothers were killed on d 35 of pregnancy, while the minipumps were still active by overdose of sodium pentobarbitone. Maternal blood was collected by cardiac puncture and centrifuged at 2500 rpm for 15 min at 4 C, then plasma was recovered and stored at −20 C.

Plasma IGF-I and IGF-II proteins were dissociated from their binding proteins (IGFBPs) by size exclusion high pressure liquid chromatography performed at pH 2.5, as previously described (42, 43). From each acidified plasma sample, four fractions were eluted from the column, and fraction 1, which contained only IGFBPs, and fraction 3, which contained only the IGFs, were collected for later analysis. The IGF fraction 3 was analyzed by specific RIAs for IGF-I and IGF-II concentrations as previously described (42, 44).

Recombinant human IGF-I and IGF-II (GroPep Pty. Ltd.) were used as standards and for preparation of radiolabeled ligands. IGF-I was measured by RIA using rabbit antihuman IGF-I (MAC Ab 89/1; GroPep Pty. Ltd.) at a final dilution of 1/60,000 and a monoclonal mouse antirat IGF-II antibody (kind gift from Dr. K. Nishikawa, Kanazawa Medical University, Ishikawa, Japan) was used at a final concentration of 1/500 to measure IGF-II by RIA. Cross-reactivity of IGF-II in the IGF-I RIA was less than 1% (44) and that of IGF-I in the IGF-II RIA was less than 2.5% (45). Both IGF-I and IGF-II amino acid sequences are remarkably conserved across species. Guinea pig IGF-I and IGF-II have previously been shown to have 100% amino acid sequence identity to those of human (46, 47), whereas guinea pig IGF-II has only one amino acid different to that of the rat (48). We have previously reported that the recoveries of IGF-I and IGF-II are more than 95% for these assays (28). The minimal detectable concentrations of IGF-I and IGF-II were 6.64 and 9.48 ng/ml, respectively. The samples were analyzed in a single RIA, where the mean intraassay coefficients of variation were 3.7 and 5.6% for IGF-I and IGF-II RIAs, respectively.

The total IGFBP binding capacity in the maternal circulation was indirectly measured as the interference of the IGFBPs in fraction 1 in the IGF-I RIA, as previously described (42). The ratio of IGFs to IGFBPs provided an index of IGF bioavailability in the maternal circulation.

Placental histology

Mid-sagittal slices of placentae that had been fixed in 4% paraformaldehyde overnight were washed in 1% PBS, dehydrated, and embedded in paraffin wax, then 5-μm sections were stained with Masson's Trichrome (49). From each dam, one to three placentae were randomly selected for histological assessment. The cross-sectional areas of the placental interlobium (germinative region) and labyrinth (exchange region) were measured in complete mid-sagittal sections using an Olympus BH-2 microscope with ×2 objective and ×3.3 ocular lenses and video

Image analysis software (Video Pro; Leading Edge, Adelaide, Australia). The proportion (percentage) of each region in the placenta was then estimated by dividing the cross-sectional area of that region by the total mid-sagittal cross-sectional area of the placenta. An estimate of the volume of these regions was then calculated by multiplying their proportion by total placental weight.

Structure of the placental exchange region (labyrinth)

To distinguish cell types within the placental labyrinth, mid-sagittal sections of placenta were double-labeled with mouse antibodies to human vimentin (3B4; Dako, Glostrup, Denmark) and human pan cytokeratin (C2562; Sigma, Sydney, Australia) to identify fetal capillaries and trophoblast, respectively, and then stained with eosin to aid the identification of maternal blood spaces. This employed a triple layer technique for each antibody, performed sequentially. Sections were deparaffinized and brought to water. For antigen retrieval, sections were incubated at 37 C for 15 min in 0.03% protease (Sigma). Endogenous peroxidase activity was quenched by incubating with 3% hydrogen peroxide in water for 30 min. Sections were then washed in three changes of PBS for 5 min each and blocked for nonspecific binding with serum-free protein block (Dako) for 10 min without washing. 3B4 antibody diluted 1:50 with 10% normal guinea pig serum and 1% BSA was applied first and incubated overnight in a humidified chamber at room temperature. Sections were washed as above, and biotinylated goat anti-mouse IgG secondary antibody (Dako) was applied for 30 min, followed by washing. Streptavidin conjugated to horseradish-peroxidase (Rockland Immunochemicals, Pottstown, PA) was applied for 40 min, then sections were washed as above. 3B4 binding was visualized by incubating with diaminobenzidine with 2% ammonium nickel (II) sulfate (Sigma) to form a black precipitate. The process was then repeated for the second primary antibody (C2562) diluted 1:50 with PBS, 10% normal guinea pig serum, and 1% BSA, but nickel was omitted from the chromogen, leaving a brown precipitate. Negative controls used irrelevant mouse IgG in place of the primary antibodies or the primary antibody diluent on its own.

The placental labyrinth was then morphometrically analyzed, as previously described (34). Briefly, the proportions (volume density) and volumes of the labyrinthine placental components were quantitated by point counting on 10 nonoverlapping fields with random systematic sampling using an Olympus BH-2 microscope with ×20 objective and ×3.3 ocular lenses. The weight of each component was estimated by multiplying the volume density by the weight of the placental labyrinth. The surface area per gram of placental labyrinth was quantitated using intercept counting and the total surface area of syncytiotrophoblast for exchange and arithmetic mean trophoblast thickness (the layer through which substrate exchange occurs) were calculated as previously described (34).

Protein localization of IGF receptors in the placenta on d 35 of pregnancy

To determine that the placenta expressed the type 1 and 2 IGF receptors at the time of treatment we localized them in placental sections from the cohort of guinea pigs that were killed on d 35 of pregnancy in which circulating IGFs had been quantified. Mid-sagittal slices of placentas were immuno-labeled with rabbit antibodies raised against human IGF1R (N-20, diluted 1:20; Santa Cruz Biotechnology, Santa Cruz, CA) and IGF2R (a kind gift from Dr. Carolyn Scott, Kolling Institute of Medical Research, Sydney, Australia; diluted 1:100). This employed a triple layer technique for each antibody performed on serial placental sections, as described above. Negative controls used irrelevant mouse IgG in place of the primary antibodies or the primary antibody diluent on its own.

Plasma metabolite and hormone concentrations

Maternal and fetal plasma glucose (glucose HK assay kit; Roche Diagnostics, Mannheim, Germany), free-fatty acids (WAKO NefaC free fatty acid kit; NovoChem, Nieuwegein, The Netherlands), cholesterol (cholesterol CHOD-PAP assay kit; Roche Diagnostics), and triglycerides (triglycerides assay kit; Roche Diagnostics) were quantified with enzymatic assay kits using a COBAS Mira automated centrifugal analyzer (Roche Diagnostics). Maternal and fetal plasma α-amino nitrogen concentrations were determined using the β-naphthoquinone sulfonate colorimetric assay as previously described (50). Maternal plasma estradiol (Ultra-Sensitive Estradiol; Diagnostic Systems Laboratories, Houston, TX) and progesterone concentrations (progesterone assay kit; Diagnostic Systems Laboratories) were quantified with RIA kits.

Statistics

To assess differences in fetal weight distribution between treatments, χ^2 tests were performed using Microsoft Excel. All other data were analyzed using SPSS version 13 (SPSS, Chicago, IL). To assess differences in maternal weight gain, repeated measured ANOVA with Bonferroni *post hoc* tests were performed. To assess differences in maternal body composition, general linear model univariate ANOVA with Bonferroni *post hoc* tests were performed. To assess differences in fetal and placental parameters, linear mixed model repeated measures ANOVA with Bonferroni *post hoc* tests were performed with the mother as a subject and the fetus or placenta as the repeated measure. The number of viable pups per litter were used as a covariate when required. Data are expressed as mean ± SEM or estimated marginal mean ± SEM as required. Data were considered statistically significant when $P < 0.05$.

Results

Exogenous maternal IGF treatment increases maternal plasma IGF-I and IGF-II

To determine the concentration of IGFs we achieved in the maternal circulation in response to this treatment, an additional cohort of guinea pigs was killed on d 35 of pregnancy, while the minipumps were still active. Exogenous IGF-I increased maternal plasma IGF-I by 340% ($P = 0.001$) and reduced that of IGF-II by 45% ($P = 0.008$; Fig. 1). Exogenous IGF-II did not alter plasma IGF-I concentrations but increased plasma IGF-II by 240% ($P = 0.004$; Fig. 1). In addition, the total apparent IGFBP activity in maternal plasma was not altered by exogenous IGF. Maternal IGF-I treatment increased the ratio of IGF-I to IGFBPs in plasma by 230% ($P = 0.004$), whereas IGF-II increased the ratio of IGF-II to IGFBPs in plasma by 125% ($P = 0.04$; Fig. 1).

IGF receptor proteins are expressed by the guinea pig placenta during the treatment

To establish that IGF1R and IGF2R are expressed by the guinea pig placenta during the IGF treatment, immunolabeling was performed on guinea pig placenta recovered from vehicle-treated mothers killed on d 35 of pregnancy (Fig. 2). IGF1R and IGF2R were ubiquitously expressed by the guinea pig placenta, with profuse cytoplasmic staining observed in trophoblast and fetal endothelium of the labyrinth and trophoblast of the interlobium (Fig. 2, A and C). Both IGF receptor proteins were concentrated on the apical surface of trophoblast within large maternal blood sinusoids and within maternal blood spaces (Fig. 2, B and D).

Exogenous maternal IGF-II, but not IGF-I, enhances development of the placental exchange region (labyrinth)

IGF treatment in early to mid-pregnancy did not alter placental weight in late gestation (Table 1). However, there was a 17% difference in placental weight between IGF-I- and IGF-II-treated mothers ($P = 0.039$). Exogenous IGF-II increased placental labyrinthine cross-sectional area by 28% ($P = 0.005$) but not that of the interlobium (Fig. 3, A–C, and

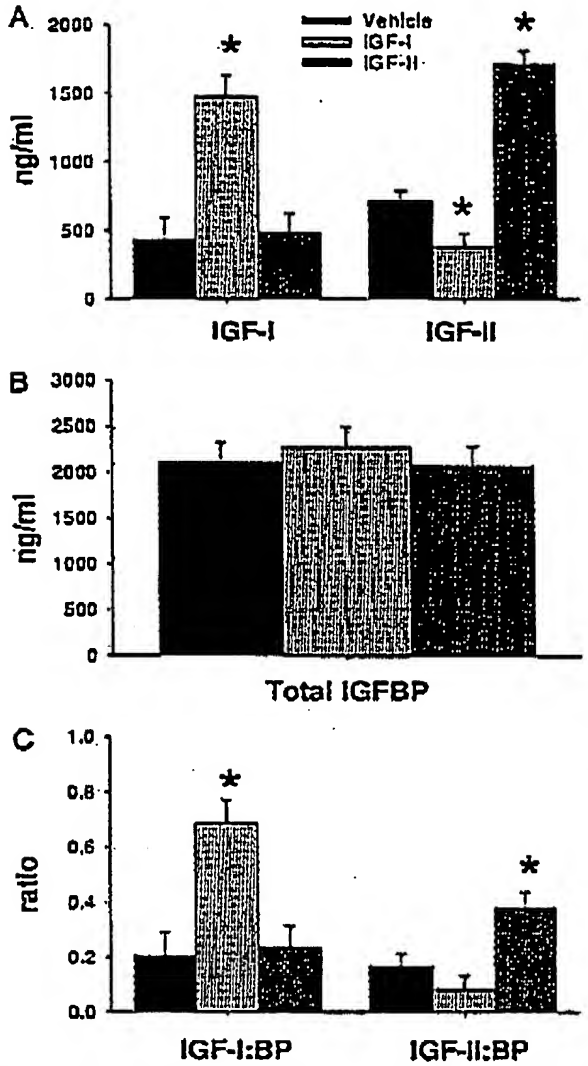

FIG. 1. The effect of exogenous maternal IGFs on maternal circulating IGF-I, IGF-II (A), and total IGFBP (B) concentrations and bioavailability of IGFs in the circulation indicated by IGF to IGFBP ratios (C) during treatment on d 35 of pregnancy. Data are from three to six mothers per treatment, and values are expressed as means ± SEM. *Asterisks* denote a statistically significant difference compared with the vehicle group, $P < 0.034$.

Table 1). The ratio of labyrinth to interlobium was increased by IGF-II (+37%, $P = 0.054$). IGF-II increased the proportion of the placenta comprised of labyrinth (+9%, $P = 0.0003$) and reduced that composed of the interlobium (−24%, $P = 0.0003$) (Table 1). IGF-II also increased the volume of placental labyrinth (+28%, $P = 0.027$) but did not alter that of the interlobium (Table 1). Maternal IGF-I treatment did not alter any placental parameter (Table 1).

To examine placental labyrinthine development in response to earlier maternal IGFs in more detail, structural correlates of placental function were quantified. Maternal IGF treatment did not alter the proportions of the placental labyrinth composed of trophoblast, maternal blood spaces, or fetal capillaries (Fig. 4A). IGF-II increased the volume of trophoblast (+29%, $P = 0.015$) and that of maternal blood spaces (+46%, $P = 0.035$) within the placental labyrinth (Fig. 4B). The total surface area of trophoblast functioning in exchange was also increased by IGF-II (+39%, $P = 0.037$, Fig. 4C). There was no effect of IGF treatment on syncytiotrophoblast barrier thickness (vehicle, 4.7 ± 0.2 μm; IGF-I, 4.8 ± 0.2 μm; IGF-II, 4.4 ± 0.2 μm). Maternal IGF-I treatment did not affect any placental labyrinthine structural parameter measured.

Exogenous maternal IGFs increase fetal survival

Maternal IGF treatment did not affect total litter size (Table 2). However, the number of resorptions was reduced by IGF-I (−77%, $P = 0.009$) and IGF-II (−60%, $P = 0.01$), while IGF-II also increased the number of viable fetuses (+25%, $P = 0.034$) near term (Table 2). Maternal IGFs did not alter the ratio of females to males (Table 2).

Exogenous maternal IGFs increase fetal growth with IGF-specific effects on fetal body composition

Maternal IGF-I and IGF-II treatment in early to mid-pregnancy increased fetal weight near term by 17% ($P = 0.002$) and 11% ($P = 0.042$), respectively (Table 3). Both maternal IGF treatments significantly skewed the fetal weight distribution to the right (both $P < 0.0005$; Fig. 5A). The percentage of fetuses heavier than 81 g was 5% in controls, 37% in IGF-I, and 19% in IGF-II-treated animals (Fig. 5A). IGF-I treatment increased fetal crown-to-rump length by 9% ($P = 0.014$), as well as abdominal circumference by 10% ($P = 0.05$). IGF-I increased the fetal weight to placental weight ratio by 29% (vehicle, 14.82 ± 0.86; IGF-I, 19.14 ± 0.73; IGF-II, 16.18 ± 0.65; $P < 0.01$). Fetal weight correlated positively with placental weight across all treatments ($r = 0.27$, $P = 0.026$) and within each of the IGF-I and IGF-II treatment groups ($r = 0.44$, $P = 0.042$ and $r = 0.40$, $P = 0.038$, respectively) but not in vehicle-treated dams alone (Fig. 5B). Overall, fetal weight correlated positively with both the mid-sagittal cross-sectional area and the estimated total volume of the placental labyrinth ($r = 0.58$, $P = 0.009$ and $r = 0.43$, $P = 0.006$, respectively), as well as the volume of trophoblast and fetal capillaries in the placental labyrinth ($r = 0.34$, $P = 0.034$ and $r = 0.62$, $P < 0.001$, respectively).

Maternal IGF-I treatment increased fetal carcass weight (+19%, $P = 0.002$), increased the combined weights of fetal kidneys (+20%, $P = 0.028$), caecum (+24%, $P = 0.027$), total gastrointestinal tract (+13.5%, $P = 0.049$), and the combined fetal fat depots (+16%, $P = 0.028$) (Table 3). Conversely, IGF-I reduced the fractional weights of the fetal spleen (−24%, $P = 0.001$), liver (−12.5%, $P = 0.002$), and brain (−18.5%, $P = 0.004$) (Table 3). Both IGF-I and IGF-II increased the weights of the fetal retroperitoneal fat (+24%, $P = 0.004$; +18%, $P = 0.031$, respectively) and combined fetal muscle mass (+22%, $P = 0.008$; +19%, $P = 0.024$, respectively; Table 3). IGF-I and IGF-II also increased the fetal triceps absolute (+29%, $P = 0.001$; +24%, $P = 0.01$, respectively) and relative weights (both +16%, $P < 0.03$, Table 3). Body composition of male

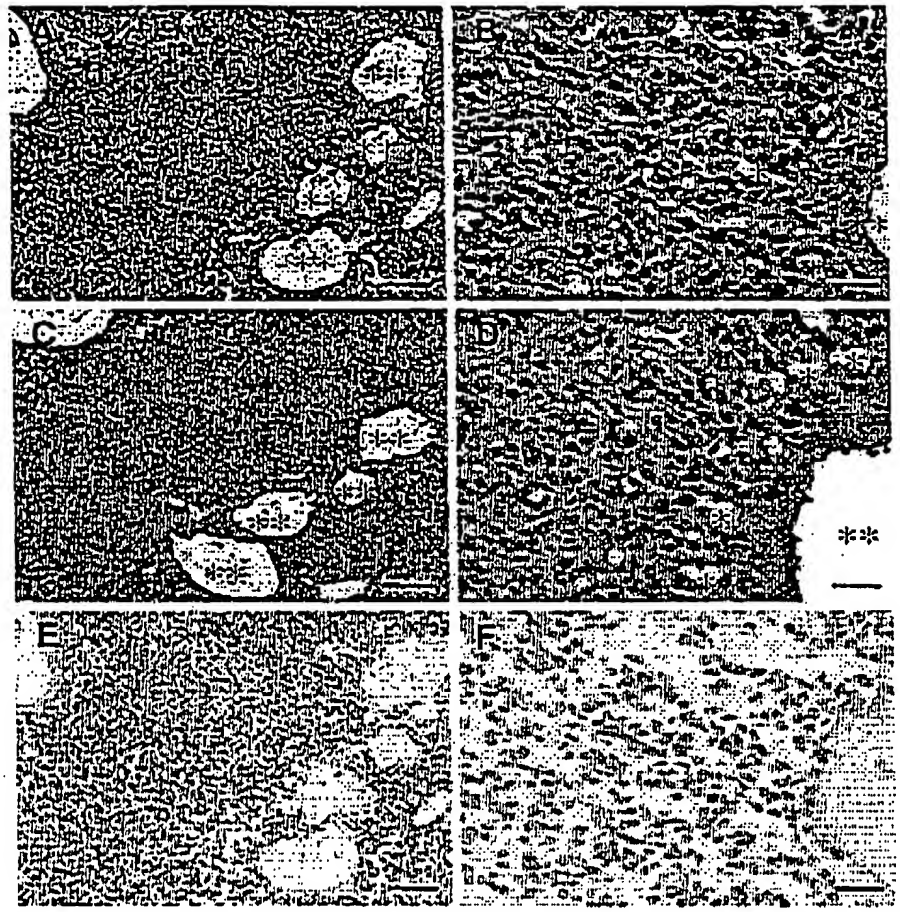

FIG. 2. Representative mid-sagittal serial sections of placentae on d 35 of pregnancy immunolabeled for the type 1 (A and B) and type 2 (C and D) IGF receptors. Representative negative control placental sections displayed (E and F). *Two asterisks* indicate maternal blood sinusoids and *single asterisks* indicate maternal blood spaces. *Scale bars*, 400 μm (A, C, and E) and 40 μm (B, D, and F).

and female fetuses was similar and was similarly affected by maternal IGF treatment (data not shown).

Exogenous maternal IGFs increase concentrations of amino acids in the fetal circulation

Maternal IGF-I and IGF-II treatment increased fetal circulating amino acid concentrations (+196%, $P = 0.026$ and +137%, $P = 0.029$, respectively) and maternal IGF-I reduced fetal circulating cholesterol concentrations (−30%, $P = 0.049$) near term (Fig. 6A). There was no effect of treatment on fetal plasma glucose, triglyceride, or free fatty acid concentrations (Fig. 6A).

Exogenous maternal IGF-I, but not IGF-II, alters maternal body composition

Weight gain and body composition analyses were performed to determine whether exogenous IGFs affected the mother. Both exogenous maternal IGF-I and IGF-II did not alter maternal weight gain during or after IGF treatment (Fig. 7), nor total body and lean body mass near term (Table 4). IGF-I reduced maternal interscapular fat depot weight (−25%, $P = 0.028$) and the fractional weights of the perirenal (−32%, $P = 0.05$), retroperitoneal (−33%, $P = 0.037$), and interscapular fat (−28%, $P = 0.01$; Table 4). IGF-I reduced the absolute and fractional weights of the combined adipose depot weights in the mother by approximately 30%, ($P = 0.016$ and $P = 0.007$, respectively). IGF-II did not alter the absolute or relative weights of any maternal organ or tissue examined.

Exogenous maternal IGF treatment does not alter maternal circulating metabolite concentrations

Maternal IGF treatment did not alter circulating concentrations of glucose, free fatty acids, amino acids, triglycerides, or cholesterol in the mother near term (Fig. 6B).

Exogenous maternal IGF treatment and maternal circulating hormone concentrations

To determine whether treatment of the mother during early to mid-pregnancy with IGFs altered maternal circulating estradiol (Fig. 7C) and progesterone (Fig. 7D), their concentrations were determined on d 35 of pregnancy in the additional cohort of guinea pigs in which the plasma IGF and IGFBP concentrations were determined as described above. Treating the mother during early to mid-pregnancy with IGF-I doubled circulating maternal estradiol concentrations

TABLE 1. Effect of maternal IGF treatment on placental structure near term

	Vehicle	IGF-I	IGF-II
Placental weight (g)	4.03 ± 0.29^{a,b}	4.11 ± 0.24^{a}	4.84 ± 0.22^{b}
Cross-sectional area labyrinth (mm^2)	98.9 ± 3.6^{a}	112.3 ± 8.9^{a}	126.6 ± 8.3^{b}
Cross-sectional area interlobium (mm^2)	35.6 ± 3.8	32.8 ± 4.3	30.4 ± 2.8
Labyrinth:interlobium	3.10 ± 0.43	3.60 ± 0.44	4.23 ± 0.35
Proportion labyrinth (%)	73.6 ± 1.2^{a}	77.0 ± 1.1^{a,b}	80.5 ± 1.1^{b}
Proportion interlobium (%)	26.4 ± 1.2^{a}	22.4 ± 1.1^{a,b}	19.6 ± 1.1^{b}
Volume labyrinth (cm^3)	3.34 ± 0.26^{a}	3.26 ± 0.23^{a}	4.26 ± 0.23^{b}
Volume interlobium (cm^3)	1.21 ± 0.09	0.95 ± 0.09	1.08 ± 0.08

Data are expressed as mean ± SEM from seven to nine dams per treatment with one to three placentae randomly selected for histological analysis.

Different superscripts denote differences between groups, *a* vs. *b*, $P < 0.039$.

in late pregnancy, although this was not quite significant ($P = 0.078$). IGF-I treatment did not alter mid or late pregnancy circulating progesterone concentrations. Exogenous maternal IGF-II during early to mid-pregnancy increased circulating estradiol concentrations (+150%) in mid-pregnancy and progesterone concentrations in mid (+53%) and late (+83%) pregnancy in the mother; however, these also did not reach statistical significance ($P > 0.08$) (Fig. 7, C and D).

Discussion

The present study demonstrates for the first time that administration of IGF-II to the mother in early to mid-pregnancy increases placental structural and functional capacity by increasing the volume and surface area of the exchange region of the placenta near term, whereas IGF-I has no effect on the placenta. IGF-I, in contrast, reduced maternal adiposity late in pregnancy, whereas IGF-II did not affect maternal body composition. Importantly, however, maternal treatment with either IGF in early to mid-pregnancy substantially reduced fetal resorptions, increased fetal weight, and increased fetal circulating amino acid concentrations near term. Furthermore, administration of IGF-II also increased fetal viability in late pregnancy. This suggests that maternal IGF abundance, particularly that of IGF-II, during the period of early placental growth and development may determine in part the margin of safety between placental capacity to deliver, and fetal demand for, substrates throughout pregnancy.

Specifically, in the current study, administration of 1 mg/kg·d IGFs increased the abundance of maternal circulating IGF-II and IGF-I by 2.5- to 3.4-fold, during early to mid-pregnancy. The concentration of free IGF to IGFBP ratio in the maternal plasma, and hence bioavailable IGF, was also substantially increased. Similar IGF treatment of guinea pigs during early to mid-pregnancy increased placental weight at mid-gestation (41), which was not sustained to near term in the current study. Importantly, however, the functional capacity of the placenta, as indicated by the mid-sagittal cross-sectional area, proportion and volume of the region devoted to exchange (labyrinth) were increased late in gestation, by prior maternal IGF-II treatment. Furthermore, although the composition of this exchange region of the placenta was unaltered by earlier maternal IGF treatment, the total volume of trophoblast and maternal blood spaces, as well as the total surface area of placenta functioning in exchange were increased by IGF-II. As the labyrinth expands at the expense of the interlobium in the second half of pregnancy in the guinea

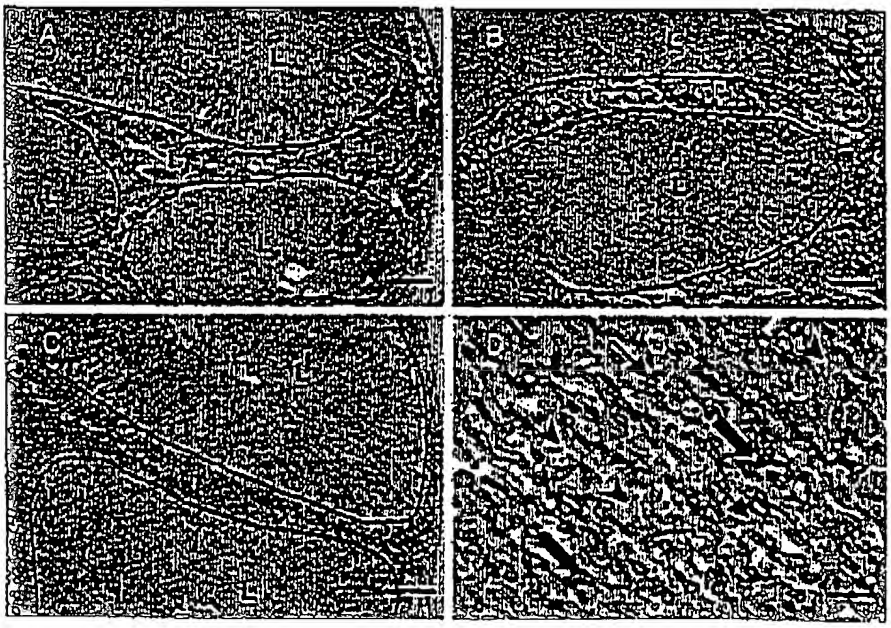

FIG. 3. The effect of exogenous maternal IGF treatment on placental structure. Representative mid-sagittal sections of near-term placentae stained with Masson's Trichrome to distinguish labyrinth and interlobium layers from mothers that had been treated with vehicle (A), IGF-I (B), or IGF-II (C) during early to mid-pregnancy. L, Labyrinth; I, interlobium. *Scale bars*, 400 μm. D, Representative mid-sagittal section of near-term placenta double-labeled and eosin stained to reveal structural components of the placental labyrinth, including fetal trophoblast (*thin arrow*), maternal blood spaces (*asterisks*), and fetal capillaries (*broad arrows*). *Scale bar*, 40 μm.

pig (30, 34, 51), together these changes in the structure of the placenta as a result of earlier exogenous maternal IGF-II are suggestive of a more mature placenta and would be expected to increase placental transport capacity. In contrast, maternal exogenous IGF-I had no effect on placental structural development.

Rapid placental structural differentiation and growth occurs in early to mid gestation in all eutherian mammals. In humans and guinea pigs, trophoblasts invade deep within the uterus and its arterioles, extensively remodeling them, to permit increased maternal blood flow to the placenta (32, 52, 53). This ensures delivery of oxygen and nutrients to the placenta, and subsequently to the fetus. The sustained effects of maternal IGF-II supplementation in early to mid-pregnancy on the placenta reported here are the converse of those observed after specific deletion of IGF-II within the placenta. IGF-II is abundantly expressed by invasive trophoblasts of human (54), mouse (55), rat (56), and guinea pig placenta (57). Ablation of placenta-specific *Igf2* gene expression (P0 transcript) in mice reduced the surface area for exchange, increased the exchange barrier thickness and also impaired nutrient transport capacity of the placenta (16, 17).

Reduced maternal circulating IGF-II in mid-pregnancy, as a result of undernutrition in guinea pigs (36), is associated with similar consequences to those of placental *Igf2* gene deletion (17), with a delay and impairment in the functional maturation of the placenta and with reduced fetal growth in both mid and late gestation (37). Together these findings indicate that maternal circulating IGF-II may act in an endocrine fashion to modulate placental development, in addition to any autocrine/paracrine effects of locally produced IGF-II. We suggest that exposure to increased circulating maternal IGF-II in early to mid-pregnancy may provide a foundation of increased placental trophoblast proliferation and invasion of the uterus and its vasculature, which leads to increased volumes of both trophoblast and maternal blood spaces in the placental labyrinth in late gestation. This would be expected to increase maternal blood flow to the placenta and enhance growth of the area devoted to exchange improving placental transfer of oxygen and nutrients to the fetus from the mother. This was consistent with increased circulating fetal amino acid concentrations with earlier maternal IGF treatment, near term. Hence, maternal IGF-II supplementation presumably increased fetal growth and viability predominantly by these actions on the placenta. Current studies in our laboratory are focused on determining whether early maternal IGF treatment increases placental transport of nonmetabolizable analogs of glucose and amino acids in the fetal circulation and tissues and whether treatment affects nutrient partitioning in the mother.

Supplementing the mother during early to mid-pregnancy with either IGF had a sustained positive effect on fetal weight, length, and girth near term, which is consistent with the anabolic effects on the fetus seen at mid-pregnancy after similar treatment in the guinea pig (41). The increased fetal weight observed with maternal IGF treatment appears to be substantially due to increased muscle mass overall and proportionately for selected muscles and perhaps enhanced fetal bone growth as indicated by increased carcass weights. This may be metabolically beneficial in later life because muscle

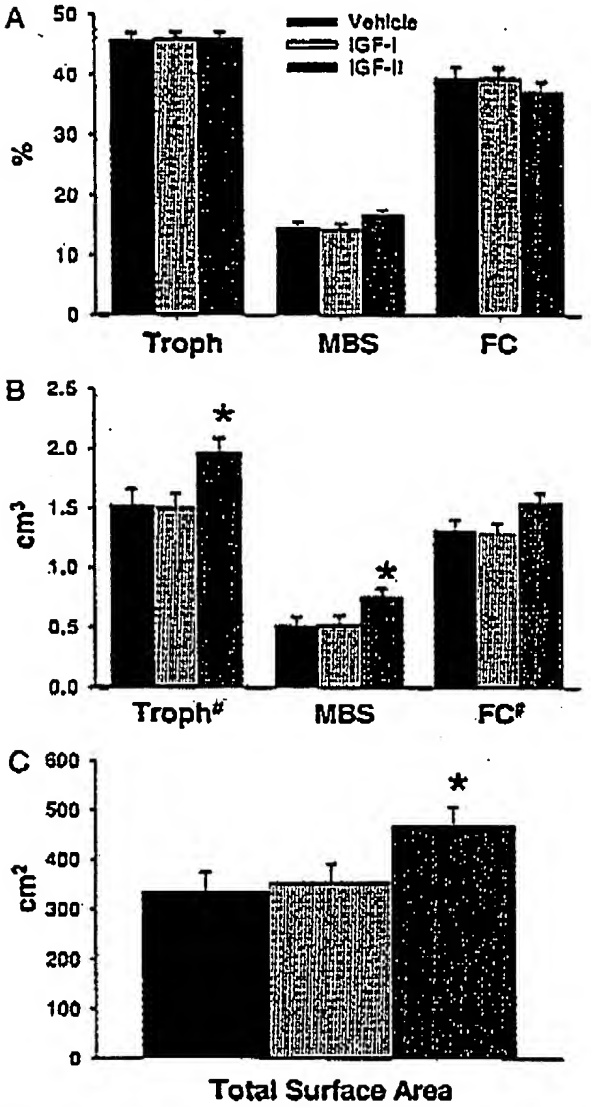

FIG. 4. The effect of exogenous maternal IGFs on structural correlates of placental exchange function near term. Proportions (A) and volumes (B) of fetal trophoblast, maternal blood spaces, and fetal capillaries in the placental labyrinth (exchange region), as well as the total surface area of syncytiotrophoblast for exchange (C). Data are from $n = 1$–3 placentae from each of seven to nine mothers per treatment. Values are expressed as means ± SEM. *Asterisks* denote a statistically significant difference compared with the vehicle group, $P < 0.05$. #, Positive correlation with fetal weight, $r > 0.34$ and $P < 0.034$.

is an important site for insulin-induced glucose uptake. Indeed, fetal growth restriction in the guinea pig, induced by maternal food restriction and accompanied by reductions in circulating maternal IGF concentrations (36), is characterized by deficits in muscle mass, increased adiposity in the fetus near term (58) and with increased blood pressure and impaired glucose and cholesterol homeostasis in adult offspring (59–61).

TABLE 2. Effect of maternal IGF treatment on litter composition and fetal dimensions near term

	Vehicle	IGF-I	IGF-II
Dams	7	7	9
Fetuses	19	22	30
Females/males	9/10	12/10	14/16
Total litter	3.42 ± 0.1	3.38 ± 0.1	3.67 ± 0.1
Number viable	2.73 ± 0.2[a]	3.27 ± 0.9[a,b]	3.40 ± 0.2[b]
Number resorbing	0.68 ± 0.1[a]	0.09 ± 0.1[b]	0.27 ± 0.1[b]

Data are expressed as mean ± SEM.
Different superscripts denote significant differences between groups, $P < 0.05$.

The present study suggests that increased maternal IGF-I and IGF-II abundances during early to mid-pregnancy promote fetal growth and viability near term by multiple mechanisms. In addition to direct effects of IGF-II on placental structural development, which in the current study were positively associated with fetal weights, the IGFs may increase nutrient transporter expression (20–22) and/or placental vasodilation (26), which would allow for more substrate to be delivered to the fetus for its growth. The IGFs may also influence placental metabolism and function, which, in turn, may drive major physiological adaptations to pregnancy in the mother, including the development of insulin resistance to divert nutrients to the conceptus (62–64). This has been attributed to placental production of hormones including estrogen, progesterone, and placental lactogen (64, 65) that reduce maternal insulin secretion (64, 65) and antagonize the effects of insulin on maternal tissues, including fat deposition (65). Treatment of the mother with IGF-II enhanced placental weight in mid-pregnancy (41) and is accompanied by elevated maternal circulating estradiol and progesterone concentrations, although these were not significant. This would be expected to amplify insulin resistance and other adaptations such as fat deposition in the mother. Consistent with this, exogenous IGF-II during early to mid-pregnancy in guinea pigs increased maternal interscapular

TABLE 3. Effect of maternal IGF treatment on fetal weight and body composition near term

	Vehicle	IGF-I	IGF-II
Fetal weight (g)	66.62 ± 2.40[a]	77.75 ± 1.96[b]	74.03 ± 1.89[b]
Crown-rump length (cm)	14.00 ± 0.34[a]	15.28 ± 0.28[b]	14.77 ± 0.24[a,b]
Abdominal circumference (cm)	8.82 ± 0.28[a]	9.09 ± 0.23[b]	9.01 ± 0.20[a,b]
Head width (cm)	6.81 ± 0.46	7.07 ± 0.09	7.20 ± 0.07
Kidneys (g)	0.59 ± 0.04[a]	0.71 ± 0.03[b]	0.67 ± 0.03[a,b]
(% Body weight)	0.89 ± 0.04	0.92 ± 0.03	0.91 ± 0.03
Spleen (g)	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01
(% Body weight)	0.17 ± 0.01[a]	0.13 ± 0.01[b]	0.15 ± 0.01[a,b]
Liver (g)	3.71 ± 0.18	3.77 ± 0.14	3.84 ± 0.13
(% Body weight)	5.6 ± 0.2[a]	4.9 ± 0.1[b]	5.2 ± 0.1[a]
Brain (g)	2.49 ± .07	2.51 ± 0.06	2.52 ± 0.05
(% Body weight)	3.8 ± 0.2[a]	3.1 ± 0.1[b]	3.5 ± 0.1[a,b]
Total GI tract (g)	3.33 ± .014[a]	3.78 ± 0.11[b]	3.59 ± 0.10[a,b]
(% Body weight)	5.0 ± 0.1	4.9 ± 0.1	4.9 ± 0.1
Caecum (g)	0.37 ± 0.03[a]	0.46 ± .02[b]	0.40 ± 0.02[a,b]
(% Body weight)	0.56 ± 0.03	0.59 ± 0.02	0.54 ± 0.02
Total muscle (g)	0.36 ± 0.21[a]	0.44 ± 0.16[b]	0.43 ± 0.15[b]
(% Body weight)	0.56 ± 0.02	0.57 ± 0.02	0.55 ± 0.01
Triceps (g)	0.17 ± 0.01[a]	0.22 ± 0.01[b]	0.21 ± 0.01[b]
(% Body weight)	0.25 ± 0.01[a]	0.29 ± 0.008[b]	0.29 ± 0.007[b]
Total fat (g)	2.39 ± 0.11[a]	2.77 ± 0.09[b]	2.72 ± 0.08[a,b]
(% Body weight)	3.6 ± 0.1	3.6 ± 0.1	3.7 ± 0.09
Retroperitoneal fat (g)	0.63 ± 0.04[a]	0.78 ± 0.03[b]	0.74 ± 0.03[b]
(% Body weight)	0.9 ± 0.04	1.0 ± 0.03	1.0 ± 0.03
Carcass (g)	48.38 ± 2.0[a]	58.01 ± 1.6[b]	53.98 ± 1.5[a,b]
(% Body weight)	73 ± 0.8	75 ± 0.6	74 ± 0.6

Data expressed as estimated marginal means ± SEM adjusted for the number of viable fetuses per litter. Only tissues that were significantly affected by treatment are shown. *Different superscripts* denote significant differences between groups, *a vs. b*, $P < 0.05$.

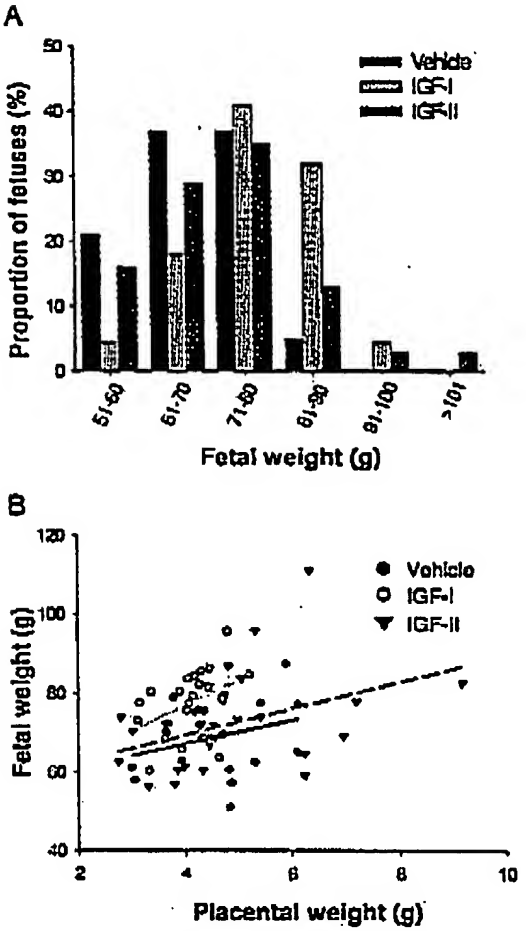

FIG. 5. The effect of exogenous maternal IGF treatment on fetal weight distribution (A) and on the association of fetal weights with placental weights (B). Each fetus from seven to nine mothers per treatment is represented.

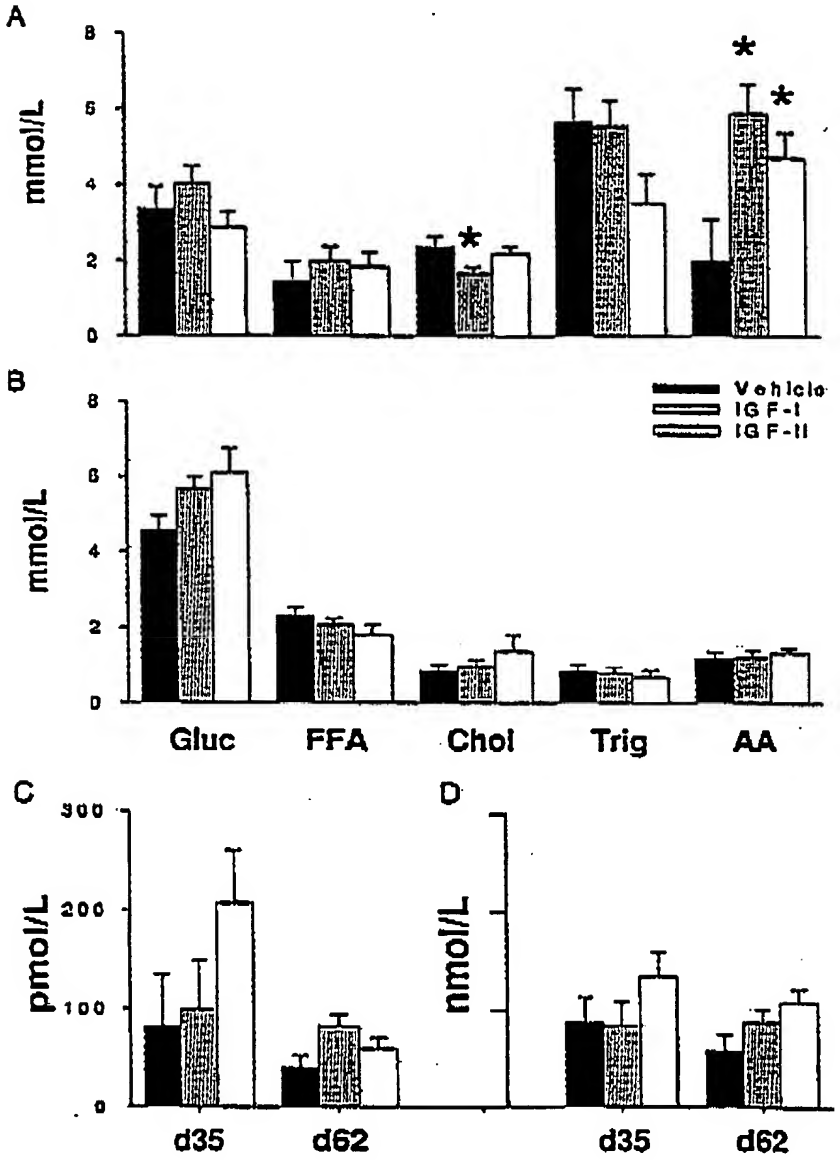

FIG. 6. The effect of exogenous maternal IGFs on circulating metabolites in the fetus (A) and mother (B) near term and estradiol (C) and progesterone (D) in the mother on d 35 and 62 of pregnancy. Fetal data are from all fetuses of six to eight mothers per treatment, and values are expressed as estimated marginal means adjusted for the number of viable pups ± SEM. Maternal data are from six to eight mothers per treatment, and values are expressed as means ± SEM. AA, Amino acids; Chol, cholesterol; d35, d 35 of pregnancy; d62, d 62 of pregnancy; FFA, free fatty acids; Gluc, glucose; Trig, triglycerides. *Asterisks* denote a statistically significant difference compared with the vehicle group, $P < 0.049$.

adiposity at mid-pregnancy (41) and there was a trend to raised maternal circulating glucose concentrations near term. These increased maternal adipose stores were depleted to normal by late pregnancy in the current study, which may have further enhanced nutrient availability for the fetus, either directly or indirectly. This suggests that IGF-II acts on the placenta to increase fetal growth, by sustainedly promoting placental development, but additionally may enhance maternal physiological adaptation to pregnancy.

The mechanism by which increased maternal IGF-I abundance in early to mid-pregnancy sustainedly promotes fetal growth is less clear. The enhanced placental weight at mid-gestation by prior maternal IGF-I treatment (41), which is no longer apparent in late gestation, may have had persistent effects on the fetus that increased fetal growth near term. In addition, unlike IGF-II, IGF-I did not increase maternal fat deposition in mid-pregnancy (41) and in fact reduced fat depot weights near term. Reduced perirenal fat weight was associated with increased maternal circulating progesterone. Reduced adiposity may reflect increased mobilization and/or reduced deposition during pregnancy, which may have increased substrate availability in the maternal circulation for fetal growth. This has been observed in growth hormone-treated pigs where maternal circulating IGF-I concentration was elevated and associated with reductions in weight of maternal backfat depots (67). Another possible explanation is that larger fetuses of IGF-I-treated dams may signal to the mother via nutrient sensors in the fetal circu-

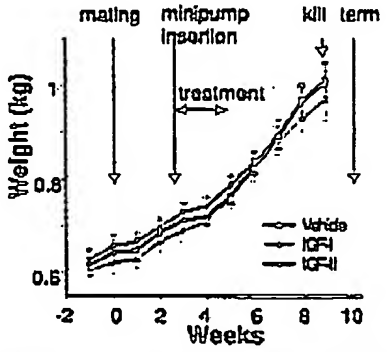

FIG. 7. The effect of exogenous IGFs on maternal weight gain during pregnancy. Female guinea pigs were weighed three times weekly during the study to determine an average weekly weight, from 1 wk before mating and during pregnancy up until kill. Minipumps were inserted on d 30 of pregnancy to deliver vehicle, IGF-I, or IGF-II for 18 d. Term, which is approximately 67–70 d of pregnancy, is denoted on the graph. Data are from seven to nine dams per treatment, and values are expressed as means ± SEM.

lation (such as IGFs and insulin), to influence placental metabolism and increase mobilization of maternal adipose tissue stores late in pregnancy.

These differential IGF effects may reflect their distinct interactions with various receptors, because IGF-I binds with high affinity to the IGF1R but negligibly to IGF2R. In contrast, IGF-II binds to both these receptors, as well as to the insulin receptor. In the current study, during mid-pregnancy, the guinea pig placenta ubiquitously expressed both IGF receptor proteins. More importantly, however, at the time of IGF treatment, IGF1R and IGF2R were localized to the apical surface of trophoblasts, within large maternal blood vessels and blood spaces of the labyrinth. In addition, insulin binding sites have previously been identified in trophoblast of the guinea pig placenta (68–70). This pattern of expression is consistent with the localization of all three receptors to placental trophoblasts in humans and rats (56, 71–77) and abundant expression of IGF1R and IGF2R in invasive trophoblast populations within the human decidua and its vasculature (75).

The specific effects of IGF-II on the placenta, which were not evident in IGF-I-treated animals, suggest that IGF-II actions on the placenta may be mediated by the insulin receptor, which has been implicated in mediating IGF-II effects on fetal growth (78) or by the IGF2R, which it binds with much greater affinity than the IGF1R. There is evidence to suggest that IGF-II acts through IGF2R to promote trophoblast migration and invasion (79), and placental angiogenesis and vascular remodeling (80). IGF-II then, indirectly at least, may enhance placental function by increasing blood supply to the placenta. In contrast, the effects of maternal IGF-I treatment are likely to have been mediated by the IGF1R, particularly because this treatment also reduced IGF-II in the mother.

In conclusion, increased maternal IGF-II in early pregnancy sustainedly promotes placental structural and functional capacity and fetal growth and viability, whereas IGF-I appears to act through the mother to enhance fetal growth to near term. This suggests sustained major and complementary roles in placental and fetal growth for increased circulating IGFs in the mother in early pregnancy.

TABLE 4. Effect of maternal IGF treatment on maternal adipose tissue weights near term

	Vehicle	IGF-I	IGF-II
Number of dams	7	7	9
Weight at d62	978 ± 23	1012 ± 34	971 ± 35
Uterus and contents	242 ± 20	250 ± 44	242 ± 38
Net body mass	736 ± 21	761 ± 62	725 ± 10
Lean body mass	711 ± 19	744 ± 62	702 ± 18
Total fat (g)	25.08 ± 2.3[a]	17.89 ± 1.6[b]	23.14 ± 1.0[a,b]
(% Body weight)	3.4 ± 0.3[a]	2.4 ± 0.3[b]	3.2 ± 0.09[a,b]
Perirenal fat (g)	5.27 ± 0.8	3.50 ± 0.5	4.72 ± 0.4
(% Body weight)	0.71 ± 0.1[a]	0.48 ± 0.08[b]	0.66 ± 0.05[a,b]
Retroperitoneal fat (g)	8.96 ± 0.9[a]	6.27 ± 0.8[b]	8.47 ± 0.6[a,b]
(% Body weight)	1.2 ± 0.1[a]	0.86 ± 0.1[b]	1.2 ± 0.06[a]
Interscapular fat (g)	10.85 ± 0.9[a]	8.11 ± 0.6[b]	9.06 ± 0.4[a,b]
(% Body weight)	1.5 ± 0.1[a]	1.1 ± 0.05[b]	1.4 ± 0.06[a]

Data expressed as means ± SEM. Only tissues that were significantly affected by treatment are shown. Net body mass is weight at postmortem minus the uterus and contents. Lean body mass is net body mass minus total fat. Tissue weight was calculated as a percentage of net body mass. *Different superscripts* denote significant differences between groups, [a] vs. [b], $P < 0.05$.

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Address all correspondence and requests for reprints to: Claire T. Roberts, Research Center for Reproductive Health, Discipline of Obstetrics and Gynecology, University of Adelaide, Adelaide, South Australia, Australia 5005. E-mail: claire.roberts@adelaide.edu.au.

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